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Inhibition of NF-κB activation by vitamin E derivatives

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Summary-Nuclear factor κB (NF-κB) is believed to play an important role in the activation of a human immunodeficiency virus (HIV) which causes acquired immunodeficiency syndrome (AIDS). Recent findings suggesting an involvement of reactive oxygen species in signal transduction pathways leading to NF-κB activation have ensured the possible clinical use of antioxidants in blocking HIV activation. The present study examined the effects of vitamin E derivatives on the tumor necrosis factor-α (TNF-α) induced NF-κB activation. Incubation of human Jurkat T cells with vitamin E acetate or α-tocopheryl succinate (10 μM to 1 mM) exhibited a concentration dependent inhibition of NF-κB activation. α-Tocopherol or succinate at these concentrations had no apparent effects. 2,2,5,7,8-Pentamethyl-6-hydroxychromane (PMC) was extremely effective, causing complete inhibition of NF-κB activation at 10 μM. Oct-1 binding activity was inactivated by α-tocopheryl succinate whereas other derivatives had no effects, suggesting that the effects of α-tocopheryl succinate are not specific to NF-κB. HPLC measurements demonstrated that treatment of cells with TNF-α had no effects on cellular α-tocopherol, but vitamin E acetate treatment increased the α-tocopherol content. Cell viability was not affected by any of the vitamin E derivatives. These results indicate a possible use of vitamin E derivatives in AIDS therapeutics. © 1993 Academic Press, Inc.

Acquired immunodeficiency syndrome (AIDS) results from infection with a human immunodeficiency virus (HIV) that eventually destroys a subset ($CD4^+$) of helper T lymphocytes, resulting in enhanced susceptibility to opportunistic infection and neoplasm. The long terminal repeat (LTR) region of HIV proviral DNA contains two binding sites for the transcription-enhancing factor, nuclear factor κB (NF-κB) which appears to play a key role in HIV activation (1-4).

As reactive oxygen species (ROS) have been shown to be involved in the signal transduction pathways which lead to the activation of NF-κB and subsequent HIV transcription, a possible therapeutic use of antioxidants for preventing HIV activation has been suggested (5-9; see 10 for recent review).

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Some of the natural antioxidants have been shown to inhibit NF-κB activation, including *N*-acetylcysteine (6,7) and α-lipoic acid (11).

Vitamin E is a well known natural lipophilic antioxidant which protects membranes from lipid peroxidation (12,13). Although its preventative role in HIV activation has not yet clearly determined (14), it is one of the most logical natural antioxidants to be examined in terms of its effect on NF-κB activation. In the present study, the effects of vitamin E and its derivatives: α-tocopherol, vitamin E acetate, α-tocopheryl succinate and 2,2,5,7,8-pentamethyl-6-hydroxychromane (PMC) [see Fig. 1 for structures] on tumor necrosis factor-α (TNF-α) induced NF-κB activation were investigated in Jurkat T cells.

Materials and Methods

Cell line and cell culture

Jurkat T (human lymphoma) cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 1% (w/v) penicillin/streptomycin, 1% sodium pyruvate and 1% glutamine (University of California San Francisco Cell Facility). Cells were plated at a density of 1 x 10^6 cells/ml. Recombinant *E. coli*-derived human TNF-α was kindly provided by Genentech Inc. (South San Francisco, CA). TNF-α (25 ng/ml) was added and cells were incubated for 4 hrs in an atmosphere of 5% CO_2 in air humidified at 37°C, followed by nuclear extraction.

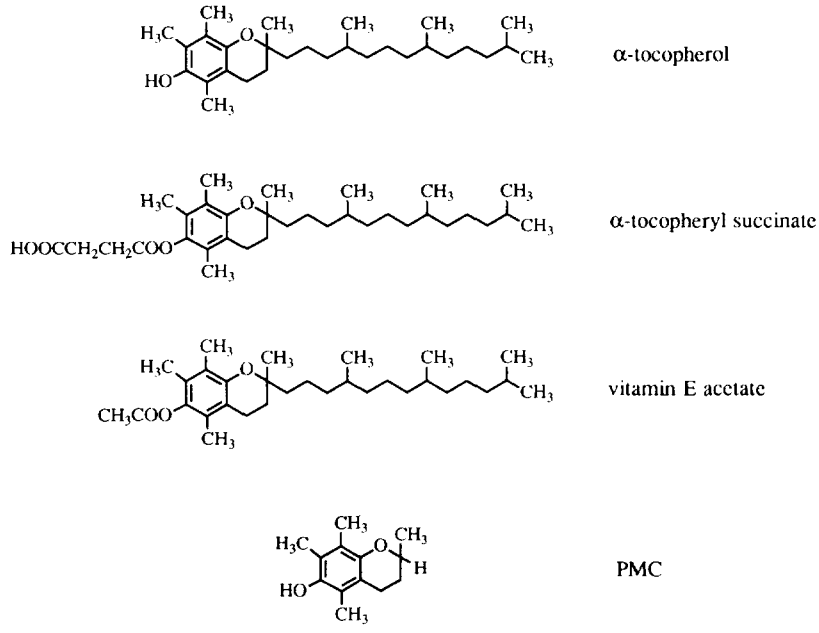

Fig. 1. Structures of α-tocopherol, α-tocopheryl succinate, vitamin E acetate and PMC.

In order to examine the effects of vitamin E derivatives, various concentrations of *d*-α-tocopherol (Henkel Corporation) *d*-α-tocopheryl succinate (Sigma Chemical Company), vitamin E acetate (Henkel), PMC (gift from the Eisai Company, Tokyo, Japan) or succinate dissolved in ethanol were added 30 min before the addition of TNF-α.

Cell viability was determined by the Trypan-Blue exclusion method.

Nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from 1 x 10^6 cells essentially as previously described (6,11) with slight modifications. Cells were harvested, centrifuged for 10 min at 1,200 rpm, washed in 1 ml of ice-cold PBS, and centrifuged for 15 sec at 14,000 rpm in an Eppendorf Brinkman-5412 centrifuge at 4°C. Cells were pelleted and washed once in 0.4 ml of buffer A [10 mM Hepes, pH 7.8; 10 mM KCl; 2 mM $MgCl_2$; 1 mM dithiothreitol (DTT); 0.1 mM EDTA; 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml antipain (Sigma) and 5 μg/ml leupeptin (Sigma)] and incubated on ice for 15 min. Then 25 μl of a 10% Nonidet P-40 solution (Sigma) were added, and cells were vigorously mixed for 15 sec and then centrifuged for 30 sec at 14,000 rpm. Pelleted nuclei were resuspended in 40 μl of buffer C [50 mM Hepes, pH 7.8; 50 mM KCl; 300 mM NaCl; 0.1 mM EDTA; 1 mM DTT; 0.1 mM PMSF; 10% (v/v) glycerol], mixed for 20 min, and centrifuged for 5 min at 14,000 rpm at 4°C. The supernatant containing the nuclear proteins was harvested, protein concentration determined and stored at -80°C.

EMSAs were performed essentially as described earlier (15,16). Binding reaction mixtures (20 μl) containing 1 to 5 μg protein of nuclear extract, 1 μg poly(dI-dC) (Pharmacia), ^{32}P-labeled probe, 50 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 2% (v/v) glycerol and 10 mM Tris-HCl (pH 7.5) were incubated for 20 min at 25°C. Proteins were separated by electrophoresis through a native 6% polyacrylamide gel in a running buffer of 12.5 mM Tris borate, 0.25 mM EDTA (pH 8.0), followed by autoradiography. NF-κB and oct-1 probes (Oncogene Science) were labeled with [α-^{32}P]dATP (ICN Biomedicals) using Klenow Fragment (Pharmacia) and purified using a NAP-5 column (Pharmacia) in 100 mM NaCl, 1 mM EDTA and 10 mM Tris-HCl (pH 7.5).

HPLC measurements

α-Tocopherol content was determined using the HPLC method described by Lang et al. (17). Cell suspensions was mixed with ethanol and hexane, then subjected to low-speed centrifugation. A portion of the hexane supernatant was removed, dried under nitrogen, and then redissolved in ethanol. The reduced form of α-tocopherol was quantitatively determined by HPLC using a C-18 reverse phase column with electrochemical detection.

Results and Discussion

Incubation of Jurkat T cells (1 x 10^6 cells/ml) with 25 ng/ml TNF-α resulted in an appearance of the NF-κB band. As shown in Fig. 2, thirty-min pre-incubation of cells with 10 μM - 1 mM α-tocopheryl succinate or vitamin E

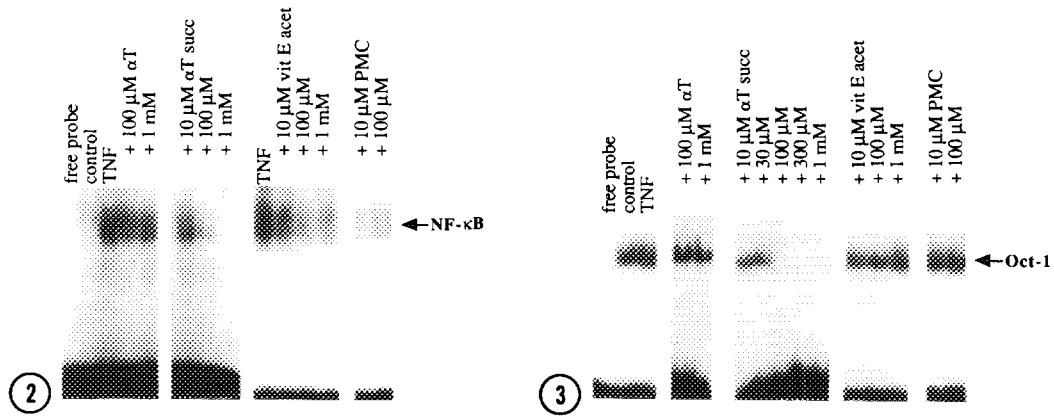

Fig. 2. Effects of vitamin E derivatives on NF-κB activation induced by TNF-α. Jurkat T cells (1 x 10^6 cells/ml) were incubated for 30 min with various concentrations of α-tocopherol and derivatives followed by incubation with TNF-α (25 ng/ml) for 4 hrs.

Fig. 3. Effects of vitamin E derivatives on oct-1 DNA binding activity. Jurkat T cells (1 x 10^6 cells/ml) were incubated for 30 min with various concentrations of α-tocopherol and derivatives followed by incubation with TNF-α (25 ng/ml) for 4 hrs.

acetate exhibited a concentration dependent inhibition of NF-κB activation. In contrast, α-tocopherol did not block the activation. PMC was the most potent inhibitor among the vitamin E derivatives examined as 10 μM completely blocked the activation. Each compound was added to the culture medium using ethanol as a vehicle, and the amounts of ethanol used in the present study did not affect the NF-κB activation. None of the vitamin E derivatives at these concentrations affected cell viability. Oct-1 DNA binding activity which constitutively exists in these cells was inhibited by α-tocopheryl succinate at concentrations effective in inhibiting NF-κB. In contrast, α-tocopherol, vitamin E acetate and PMC had no effects (Fig. 3). Incubation of the cells with succinate (10 μM - 1 mM) did not inhibit NF-κB or oct-1 DNA binding activity (data not shown).

HPLC measurements determined that unstimulated Jurkat T cells contain 0.45±0.04 pmoles reduced form of α-tocopherol in 10^6 cells. This value was not significantly affected by the incubation of cells with TNF-α (25 ng/ml) for 4 hrs (0.52±0.04 pmol/10^6 cells). These values represent mean±S.E. where n=9. HPLC also demonstrated that incubation of the cells with vitamin E acetate (1 mM) for 4.5 hrs significantly increased the cellular α-tocopherol content.

As the AIDS epidemic continues to be destructive (18), if not a cure, at least the development of strategies for long-term survival for HIV positive individuals is warranted. Prevention of HIV activation by maintaining integrated proviral DNA in the latently infected stage is, to a degree, a certain success for

such a purpose, and thus blocking the activation of HIV transcription at the level of NF-κB using antioxidants may lead to a long-term survival. Furthermore, the elimination of virus by antiviral agents such as reverse transcriptase inhibitors (e.g., AZT, DDC, DDI) exhibits some toxicity against the host and the use of high concentrations is not desirable. This has led to favor the idea of a combination therapy for AIDS (19). Natural compounds which can affect the HIV lifecycle are attractive in that they may support the actions of aggressive antiviral agents without a risk of toxicity. The effectiveness of a natural NAC was demonstrated earlier (5,6). In our laboratory, another natural thiol antioxidant, α-lipoic acid, was recently found to be more potent in blocking NF-κB activation than NAC (11).

Results from the present study are intriguing since, for the first time, a compound which is known as a lipophilic antioxidant was shown to be effective in inhibiting the TNF-α induced NF-κB activation, and that such a compound appears to be even more effective than NAC or α-lipoic acid. The natural form of α-tocopherol, vitamin E acetate at 100 μM and 1 mM almost completely blocked the activation, however, never caused complete inhibition. It has been shown that vitamin E acetate becomes deesterified to the biologically active antioxidant form, α-tocopherol (20). This implies that free radical processes involved in the TNF-α induced NF-κB activation, at least in part, occurs proximal to the membranes, and membrane oxidation may be an integral step in the signal transduction pathway. The ineffectiveness of direct addition of α-tocopherol in inhibiting the NF-κB activation may also imply that the membrane oxidation processes required for the cell signalling are localized in the internal compartments of cell architecture such as mitochondria where vitamin E acetate, but not α-tocopherol, can be reached before deesterification. Furthermore, observations that vitamin E content does not decrease in response to TNF-α treatment suggest that the cell possesses an efficient vitamin E recycling mechanism which has been shown to occur in many cell types (21, 22). In Jurkat cells, this may be accomplished by utilizing reduced glutathione (GSH) as Staal et al. (6) observed decreased levels of GSH in response to TNF-α exposure. On the other hand, since α-tocopherol has been demonstrated to inhibit protein kinase C (23,24) and protein kinase C has shown to directly phosphorylate IκB (25), the inhibition of this enzyme by α-tocopherol may be the mechanism of the NF-κB inactivation. In this case, our HPLC results may imply that the oxidation process involved in NF-κB activation is localized in the cytosolic compartment and membrane oxidation does not occur; α-tocopherol content thus remains unchanged.

α-Tocopheryl succinate appears to exert non-specific effects on DNA binding proteins as demonstrated by the inhibition of oct-1 DNA binding activity. Since neither succinate nor α-tocopherol alone inhibit NF-κB activation, a unique behavior exerted by the α-tocopheryl succinate structure must be required for such an action.

The ability of PMC to inhibit NF-κB activation should gain a considerable attention. The effectiveness at a low concentration (10 μM) possibly suggests that its free radical scavenging ability may not be the major mechanism of its action as radical scavenging antioxidants generally require somewhat higher concentrations for NF-κB inhibition (10). Inactivation of enzymes involved in the TNF-α signal transduction leading to NF-κB activation is an alternative explanation for these observations. In fact, we found that PMC inhibits phospholipase A_2 in human keratinocytes (26) and a role of phospholipase A_2 in TNF-α action has been suggested (27). Thus, PMC may inhibit NF-κB activation by inactivating this enzyme. On the other hand, potency of PMC against NF-κB inhibition may be due to the ability of this more hydrophilic compound to reach the critical target where it may engage in the same activity as the longer derivatives.

Further work is needed to elucidate the mechanisms of the NF-κB inhibitory actions of vitamin E acetate, α-tocopheryl succinate and PMC all of which appear to utilize different processes. Understanding such differences in the actions of vitamin E derivatives which may be governed by the different physicochemical characteristics, resulting in influencing a common biological outcome, may be important in future drug design by defining the 'structure-activity relationships at the cellular level'. The present study suggests that vitamin E acetate which is a natural, safe compound, and PMC which was demonstrated to be very effective in blocking NF-κB activation, should be considered for possible inclusion in the combination therapy for AIDS.

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