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INHIBITION OF STIMULUS-SPECIFIC NEUTROPHIL SUPEROXIDE GENERATION BY ALPHA-TOCOPHEROL

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Alpha-tocopherol but not 2-carboxy-2,5,7,8-tetramethyl-6-chromanol (trolox or CTMC) and 2,2,5,7,8 pentamethyl-6-hydroxy chromane (PMC), derivatives of α-tocopherol, inhibited the superoxide (O_2^-) generation of rat peritoneal neutrophils (RPMN) induced by phorbol 12-myristate 13-acetate (PMA). ID_{50} for neutrophils obtained from the peritoneal cavity of rat and guinea pig was about 1μM. This concentration, however, was much lower than that for the inhibition of PMA-activated phospholipid-dependent protein kinase (PKC) (ID_{50} = 30 μM). The α-tocopherol sensitive O_2^- generation was also observed in neutrophils induced by dioctanoylglycerol (diC_8) and calcium ionophore A23187 but not by formylmethionyl-leucyl-phenylalanine (FMLP), opsonized zymosan (OZ) and sodium dodecyl sulfate (SDS). The pattern of inhibition by α-tocopherol was quite similar to that of staurosporine, a specific inhibitor of PKC. The α-tocopherol content of RPMN was 12 ng/10^6 cells and a linear increase to 200 ng/10^6 cells by addition of α-tocopherol to the cell suspension corresponded with an increased inhibition of O_2^- generation. These results indicate that both the chemical structure and the content of α-tocopherol might be important factors in O_2^- generation by neutrophils.

KEY WORDS: α-tocopherol; neutrophil O_2^- generation; protein kinase C inhibitor.

Abbreviations Cyt.c, ferricytochrome c; DG, diacylglycerol; diC_8, L-α-1,2-dioctanoyl glycerol; FMLP, formyl-methionyl-leucyl-phenylalanine; GPtPMN, guinea pig peritoneal neutrophil;KRP, Krebs-Ringer phosphate; PKC, Ca^{2+}-and phospholipid-dependent protein kinase; PMC, 2,2,5,7,8 pentamethyl-6-hydroxy chromane; PMA, phorbol 12-myristate 13-acetate; O_2^-, superoxide anion; OZ, opsonized zymosan; RPMN, rat peritoneal neutrophils; SDS, sodium dodecyl sulfate; trolox, 2-carboxy-2,5,7,8-tetramethyl-6-chromanol (CTMC)

INTRODUCTION

Alpha-tocopherol, Vitamin E, has been known for many years as an antioxidant.[1,2,3] Mahoney and Azzi[4,5] found an inhibition of brain PKC by α-tocopherol *in vitro*. The inhibitory action was concentration dependent, and half maximum inhibition occurred

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at a concentration of 30 μM. In support of a proposed mechanism of PKC inhibition by α-tocopherol, it was reported that the vitamin inhibited the PMA-induced translocation of PKC from cytosol to membrane.[6] It was also found that the inhibition of PKC ran parallel to the inhibition of smooth muscle cell proliferation by α-tocopherol,[7-9] and that the inhibition of PKC in a model system using the OZ-induced chemiluminescence of cypridina luciferin analogue in polymorphonuclear cells[10] and in PMA-induced superoxide anion production by human polymorphonuclear leukocytes.[11] A similar suppression of superoxide generation by α-tocopherol was observed in macrophages isolated from rats after oral administration of α-tocopherol.[12-13] Recently many different pathways for O_2^- generation in neutrophils have been postulated, one of which depends on PMA or diacyglycerol (DG) and calcium ionophore and is inhibited by PKC inhibitors.[14-16] A second pathway involves receptor-mediated O_2^- generation, such as the FMLP- or OZ-stimulated reaction which is insensitive to PKC inhibitors but is inhibited by genistein, a tyrosine kinase inhibitor.[16-19] Another reaction is sodium dodecyl sulfate (SDS)- or arachidonate-dependent and modulates the physicochemical nature of the cell membrane.[20,21] This reaction was not inhibited by PKC inhibitors or tyrosine kinase inhibitors. Thus the role of α-tocopherol in the mechanism of inhibition of neutrophil O_2^- generation is not fully understood. In experiments described in this paper, we examined the ability of α-tocopherol to inhibit PKC-mediated processes in intact cells using the phorbol ester- or DG-stimulated respiratory burst of neutrophils as a model system and compared it with the other O_2^- generating system induced by various other stimuli, such as FMLP, Ca^{2+} ionophore A23187 and SDS. We found that α-tocopherol specifically inhibits PKC dependent O_2^- generation of neutrophils. This inhibition was found to be a consequence of the structure of α-tocopherol.

MATERIALS AND METHODS

Chemicals

A23187, Ferricytochrome c (Cyt. c), FMLP,PMA, sodium arachidonate and zymosan were purchased from Sigma Co. (St. Louis, Mo). diC_8 was purchased from Avanti Polar Lipid (USA). D-α-tocopherol was kindly donated by Eisai Co. Ltd (Tokyo). PMA,DG and FMLP were dissolved in ethanol, and the final concentration of ethanol in the reaction mixture was less than 0.5%.

Neutrophils

Guinea pig peritoneal neutrophils (GPtPMN) and RPMN were isolated from the peritoneal cavity 16 hr after intraperitoneal injection of 2% Nutrose and washed with calcium-free KRP, pH 7.4, as described by Takahashi *et al.*[22,23] Neutrophils were stimulated by 1.25×10^{-8}M FMLP, $1 - 2 \times 10^{-9}$M PMA, 1×10^{-6}M diC_8, 1×10^{-4}M SDS,200 μg/ml OZ and 1×10^{-6}M A23187 at 37°C.

Measurement of O_2^- Generation

O_2^- production was assayed by reduction of Cyt. c as described previously using a dual beam spectrophotometer (Shimadzu UV 3000) equipped with a water-jacketed cell holder and magnetic stirrer.[16,22] Briefly, the reaction was started by adding neutrophils

(1×10^6 cells/ml) at 37°C in the medium of KRP containing 10 mM glucose, 100 μM Cyt. c and 1 mM $CaCl_2$ in the presence or absence of various ligands. The change in absorbance at 550–540 nm ($A_{550-540}$)[24] was monitored continuously using a dual beam spectrophotometer (Shimadzu UV 3000).

In Vivo Phosphorylation

GPtPMN were washed three times with phosphate-free RPMI-1640 medium containing 2 mM EGTA and suspended in the same medium at a final concentration of 4×10^7 cells/ml. Neutrophils (4×10^7 cells/ml) were incubated in one ml phosphate-free RPMI at 37°C under 5% CO_2 and 95% air for 30 min, and carrier free [^{32}P]orthophosphate (1 mCi/ml) was added. After 1 hr, the cells were washed with phosphate-free RPMI 1640 and suspended in the same medium (2×10^6 cells/ml) and incubated for 10 min at 37°C. Thereafter, various concentrations of α-tocopherol were added and the reaction mixture was incubated for 2 min at 37°C. One μl/ml of 10^{-6}M PMA was then added and the mixture incubated for another 3 min. Final concentrations of α-tocopherol and PMA were 0.01–10 μM and 1 nM, respectively. The reaction was terminated by adding ice-cold 45% trichloroacetic acid solution containing 2 mM phenylmethylsulfonyl fluoride to yield a final concentration of TCA 15%. The precipitant was washed twice with ice-cold ether/ethanol (1;1) and dissolved in sodium dodecyl sulfate (SDS) sample buffer. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with a 10% gel in 0.1% SDS. The gel was stained with Coomassie brilliant blue R250, and the piece of dried gel was autoradiographed on a Kodak X-Omat film with an intensifying screen (Dupont Cronex Lightning-Plus) at –80°C.[25]

Determination of α-Tocopherol Content in Neutrophils

The α-tocopherol content of cells was determined using HPLC with an electrochemical detector according to the method of Tamai *et al.*[26] Briefly, one ml of cell suspension (1×10^6 cells) and 1 ml of tocol in ethanol were suspended in centrifuge tubes together with 1 ml of 6% pyrogallol solution in ethanol and preincubated for 2 min at 70°C. The incubation mixture was added to 0.2 ml of 60 % KOH and saponified at 70°C 30 min and then cooled with water and mixed with 2.5 ml of distilled water and 5 ml of n-hexane. The mixture was vigorously shaken for 5 min and centrifuged at 3,000 rpm for 5 min. The 4 ml hexane layer was evaporated under nitrogen gas flow at 40°C and dissolved in 50 μl of ethanol and analyzed by HPLC (Shimadzu LC-6A) with the Schimadzu electrochemical detector (L-ECD-6A) using 4.6 × 150 mm column of CLC-ODS (M). The eluents were methanol/water/$NaClO_4$ in a ratio of 100/2/7 (v/v/w).

Statistical Treatment of Results

At least three independent experiments were performed except where indicated. Results are presented as the mean value ± standard deviation (S.D.).

RESULTS AND DISCUSSION

Effect of α-Tocopherol on the stimulated Generation of $O_2^{\cdot}$

$O_2^{\cdot}$ generation of neutrophils was stimulated by various agents such as receptor binding FMLP, protein kinase C activator PMA and membrane modulator anionic

amphiphiles.[27] PMA-induced O_2^- generation in GPtPMN was strongly inhibited by α-tocopherol (Figure 1). A similar inhibition was also observed on O_2^- generation induced by diC_8, a natural stimulator of PKC (data not shown). The inhibition was concentration dependent and the ID_{50} of α-tocopherol was 1 μM (Figure 1). This inhibitory action of α-tocopherol was also observed in RPMN (Figure 2). However, OZ-induced O_2^- generation was not inhibited by α-tocopherol even at 10 μM (Figure 2). These results indicate that the sensitivity of stimulation-dependent O_2^- generation to α-tocopherol differed according to the stimulus. Therefore we compared O_2^- generation of GPtPMN induced by a number of different stimuli. Figure 3 shows the sensitivity of O_2^- generation induced by various stimuli to α-tocopherol. O_2^- generation induced by stimuli activating PKC, such as PMA, diC_8 and A23187, are inhibited by α-tocopherol but not O_2^- generation induced by other stimuli such as FMLP, OZ and SDS. The results indicate that α-tocopherol can affect O_2^- generation through inhibition of PKC *in vivo* as observed with staurosporine.[28] The ID_{50} for O_2^- generation, however, was much lower than that for the inhibition of PKC activity in a cell free system (ID_{50} = 30 μM).[4,5] The reason for the discrepancy between the two systems might be due to the accumulation

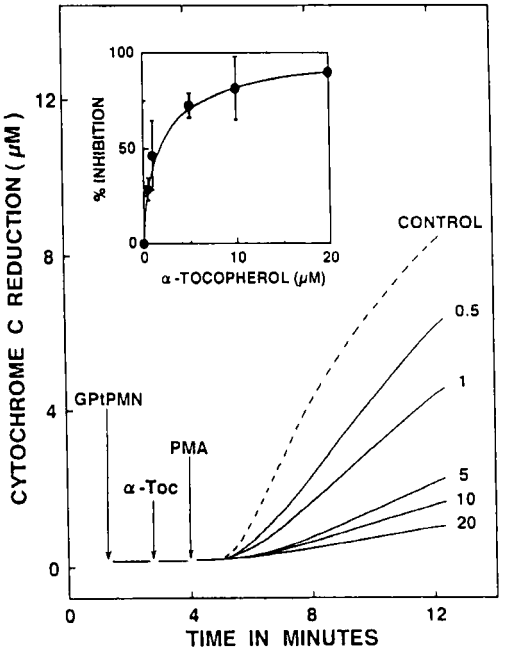

FIGURE 1. Effect of α-tocopherol on PMA-induced O_2^- generation in neutrophil. GPtPMN (10^6 cells/ml) were incubated in Krebs-Ringer-phosphate solution (KRP) (pH 7.4) containing 10 mM glucose and 1 mM $CaCl_2$ at 37°C. The total incubation volume was 2 ml and the respiratory burst was measured spectrophotometrically by monitoring cytochrome c reduction[24] Numbers show the concentrations of α-tocopherol. Concentration of PMA was 1×10^{-9}M. Inset figure shows the dose-dependent inhibition by α-tocopherol (Data are mean ± S.D. from 3 separate experiments). The % inhibition was measured 5 min after addition of PMA. α-Toc, α-tocopherol

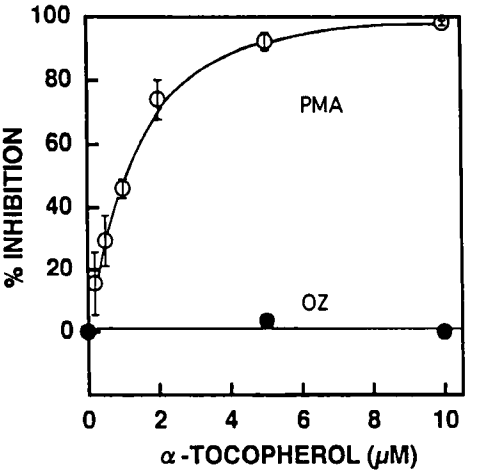

FIGURE 2. Effect of α-tocopherol on the PMA- and OZ-induced O_2^- generation of RPMN. Experimental conditions were the same as described for FIGURE 1 except that the neutrophils were 1×10^6 cells/ml of RPMN. Concentrations of PMA and OZ were 2×10^{-9} M and 200 μg/ml, respectively. Data are mean ± S.D. from 3 separate experiments. The % inhibition was measured at 5 min after the addition of PMA or OZ.

of α-tocopherol in the plasma membrane of cell suspension. In summary, PMA-, DG- or A23187-induced O_2^- generation was inhibited by α-tocopherol through the inhibition of PKC but the other stimulation-dependent O_2^- generation, which is not sensitive to PKC inhibitors, was not inhibited by α-tocopherol.

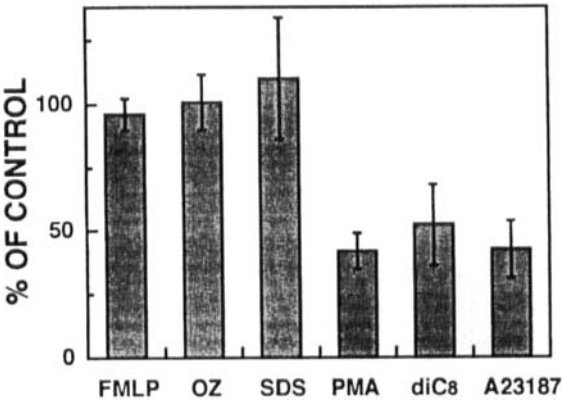

FIGURE 3. Dependence of neutrophil O_2^- generation on different stimuli and their inhibition by α-tocopherol. Experimental conditions were the same as described in FIGURE 1. The concentrations of stimulatory agents were 1.25×10^{-8} M FMLP, 200 μg/ml OZ, 1×10^{-4} SDS, 1×10^{-9} M PMA, 1×10^{-6} diC_8, and 1×10^{-6} M A23187. The concentration of added α-tocopherol was 1 μM. % inhibition was calculated 5 min after addition of stimulating agent. Data are mean ± S.D. from 3 separate experiments.

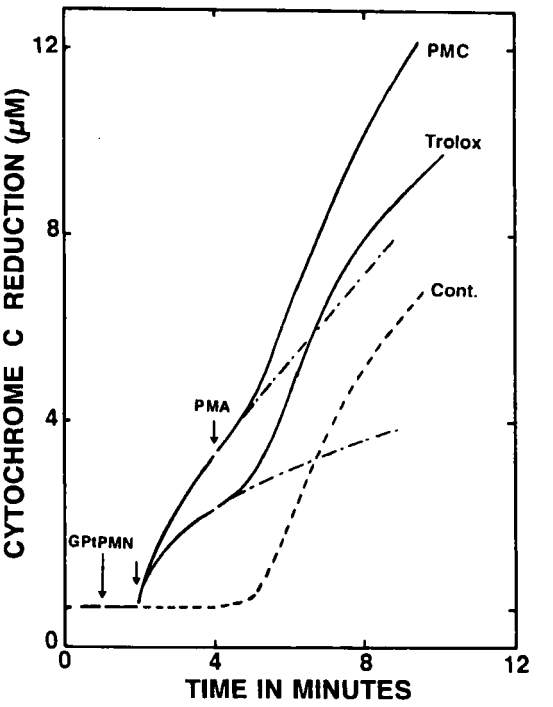

FIGURE 4. Effect of trolox and PMC on the O_2^- generation of GPtPMN. Experimental conditions were the same as described in FIGURE 1. Concentrations of Trolox and PMC were 50 μM. Cont, without addition of PMC or trolox.

Effect of Trolox and PMC on PMA-induced O_2^- Generation of Neutrophils

Azzi *et al.* observed a lack of effect of trolox, a water soluble α-tocopherol derivative on PKC activity.[5] Therefore, it was of interest to compare the effect of trolox with that of PMC, a water insoluble derivative that has no isoprenoid side chain, on the stimulation dependent O_2^- generation of neutrophils. Neutrophil O_2^- generation was slightly stimulated by trolox in the absence of stimuli and PMA-induced O_2^- generation was further increased by trolox (Figure 4). As expected, O_2^- generation induced by various stimuli, such as diC_8, A23187, SDS, OZ and FMLP, was not inhibited by trolox (data were not shown). These results agree with the results of Azzi *et al.* Quite similar effects to those of trolox were observed with PMC which like trolox has no isoprenoid side chain. However, unlike trolox the compound is insoluble in water. These results indicate that the isoprenoid side chain seems to have an important role in the inhibitory action of α-tocopherol but not related to its radical scavenging activity. However, further experiments are required to resolve the molecular mechanism of inhibition.

Relationship Between α-Tocopherol Content and PMA-dependent O_2^- Generation of Neutrophils

To confirm the idea that the content of α-tocopherol regulates stimulation-dependent O_2^- generation, the relationship between α-tocopherol content of neutrophils and

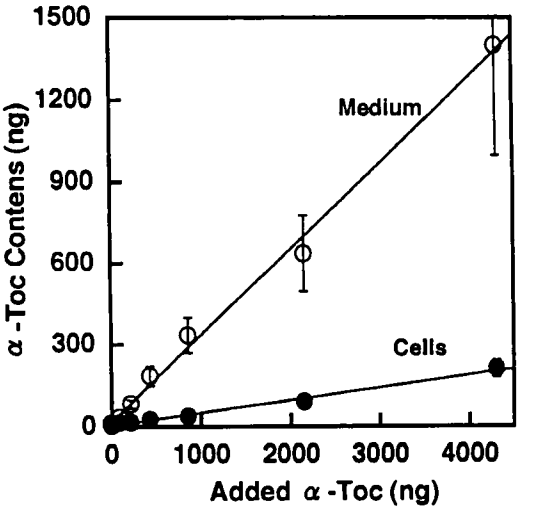

FIGURE 5. Incorporation of α-tocopherol into RPMN by addition of the vitamin to the medium. Experimental conditions were the same as described in Figure 1 except that the neutrophils were RPMN. The α-tocopherol content of neutrophils was determined by the method of Tamai *et al.*[26] after 10 min incubation at 37°C with various concentrations of α-tocopherol (Data are mean ± from 3 separate experiments). Medium, reaction medium; Cells, RPMN

PMA-stimulated $O_2^{\cdot}$ generation was observed *in vitro*. The α-tocopherol content of RPMN was about 12 ng/10^6 cells. The content of α-tocopherol was linearly increased in the cells by addition of the vitamin to the cell suspension medium (Figure 5). Moreover, the α-tocopherol incorporated into cells was quite small and most of the

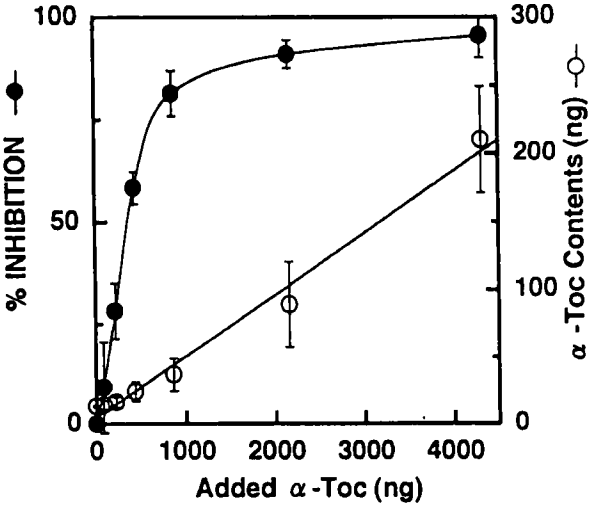

FIGURE 6. Effect of α-tocopherol on PMA-induced $O_2^{\cdot}$ generation and the vitamin content of neutrophil. Experimental conditions were the same as described in FIGURE 5 and PMA added to stimulate neutrophils was 2×10^{-9} M. (Data are mean ± from 3 separate experiments).

vitamin remained in the incubation mixture (Figure 5). In contrast to the linear incorporation into the cells, however, the rate of inhibition was not linear and 80% inhibition of the O_2^- generation was observed at about 50 ng α-tocopherol/10^6 cells (Figure 6). These results indicate that the distribution of α-tocopherol in cells is important for its PKC-mediated inhibition of neutrophil O_2^- generation.

Effect of α-Tocopherol on PMA-induced Phosphorylation of GPtPMN 47 kDa Protein

In studies of patients with autosomal chronic granulomatous disease, two cytoplasmic factors of 47 kDa ($p47^{phox}$) and 67 kDa ($p67^{phox}$) were found to be essential for O_2^- generation in neutrophils,[29] and the role of PKC in the phosphorylation of $p47^{phox}$ has been suggested to be important in the activation of NADPH oxidase.[30] Thus, the effect of the vitamin on the incorporation of [^{32}P]orthophosphate into cytoplasmic $p47^{phox}$ of GPtPMN was examined to confirm the inhibition of PMA-induced phosphorylation of cytoplasmic protein by α-tocopherol. Many cytoplasmic proteins were phosphorylated without stimulation with PMA but phosphorylation of $p47^{phox}$ was enhanced by treatment with PMA. Alpha-tocopherol (1–10 μM) inhibited the phosphorylation of these neutrophil proteins including $p47^{phox}$ in a concentration dependent manner (Figure 7). This result suggested that inhibition of phosphorylation of $p47^{phox}$ may be correlated with the inhibition of O_2^- generation in neutrophils by α-tocopherol.

The results obtained in these experiments suggested that PMA- or diC_8-induced O_2^- generation in neutrophils correlated with the activity of PKC and that the regulatory

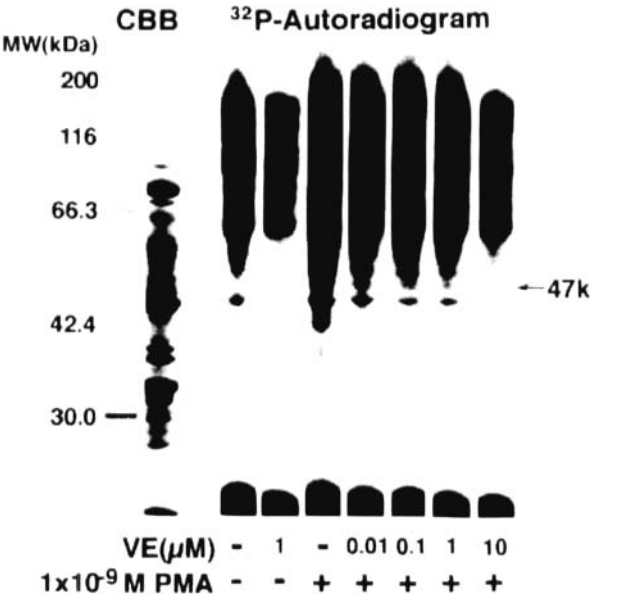

FIGURE 7. Effect of α-tocopherol on phosphorylation of neutrophil proteins stimulated by PMA. Phosphorylation was assayed by incorporation of [^{32}P]orthophosphate into the GPtPMN proteins as described in Materials and Methods.[27] PKC was stimulated by 1×10^{-9} M PMA. An arrow shows the $p47^{phox}$ (47k). VE, α-tocopherol

activity of α-tocopherol may operate via inhibition of PKC activity. However, no parallel relationship was observed between the content of α-tocopherol and inhibition of PMA-induced O_2^- generation of RPMN after addition of the vitamin. These results suggest that α-tocopherol can regulate PKC-dependent signal transducing reactions of neutrophils under certain conditions and that the distribution of α-tocopherol in the membranes may changed where it can no longer affect the PKC-dependent O_2^- generation of neutrophils. However, no direct evidence was obtained in these experiments to explain the inhibition of neutrophil O_2^- generation by α-tocopherol through a PKC mediated pathway. Moreover, trolox and PMC did not inhibit O_2^- generation. These data show that the isoprenoid side chain may have an important role in the inhibitory mechanism. Further experiments, therefore, are needed to understand the molecular mechanism of the inhibition of O_2^- generation by added α-tocopherol.

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References

1. Tappel, A.L., Brown, W.D., Zalkin, H. and Maier, V.P. (1961) Unsaturated lipid peroxidation catalyzed by hematin compounds and its inhibition by vitamin E. *Journal of the American Oil Chemists Society*, **38**, 5–9.
2. Packer, J.E., Slater, T.F. and Willson, R.L. (1979) Direct observation of a free radical interaction between vitamin E and vitamin C. *Nature*, **278**, 737–738.
3. Burton, G.W., Ingold, K.U. (1981) Antioxidant activity of vitamin E and radical chain-breaking phenolic antioxidants in vitro. *Journal of the American Chemical Society*, **103**, 6472–6477.
4. Mahoney, C.W. and Azzi, A. (1988) Vitamine E inhibits protein kinase C activity. *Biochemical and Biophysical Research Communications*, **154**, 694–697.
5. Azzi, A.M., Bartoli, G., Boscoboinik, D., Hensey, C and Szewczyk, A. (1993) α-tocopherol and protein kinase C regulation of intracellular signalling. *In*: L. Packer and Fuchs, L. (eds): *Vitamin E in health and disease*, Marcel Dekker, Inc. New York, pp371–383.
6. Boscoboinik, D., Szewczyk, A., Hensey, C. and Azzi, A. (1991) Inhibition of cell proliferation by alpha-tocopherol. Role of protein kinase C. *Journal of Biological Chemistry*, **266**, 6188–6194.
7. Boscoboinik, D., Szewczyk, A. and Azzi, A. (1991) Alpha-tocopherol (vitamin E) regulates vascular smooth muscle cell proliferation and protein kinase C activity. *Archives of Biochemistry Biophysics*, **286**, 264–269.
8. Ozer, N.K., Ploozza, P., Boscoboinik, D. and Azzi, A. (1993) d-alpha-Tocopherol inhibits low density lipoprotein induced proliferation and protein kinase C activity in vascular smooth muscle cells. *FEBS Letters*, **322**, 307–310.
9. Chatelain, E., Boscoboinik, D.O., Bartoli, G.M., Kagan, V.E., Gey, F.K. Packer, L. and Azzi, A. (1993) Inhibition of smooth muscle cell proliferation and protein kinase C activity by tocopherols and tocotrienols. *Biochimica Viophysica Acta*, **1176**, 83–89.
10. Engle, W.A., Bayrket, Y.J.L. and Yu, P. (1988) Vitamin E decreases superoxide anion production by polymorphonuclear leukocytes. *Pediatric Research*, **23**, 245–248.
11. Okano, T., Tamai, H. and Mino, M. (1991) Superoxide generation in leukocytes and vitamin E. *International Journal of Vitamin and Nutrition Research*, **61**, 20–26.
12. Sakamoto, W., Fujie, K., Handa, G., Ogihara, T. and Mino, M. (1990) In vivo inhibition of superoxide production and protein kinase C activity in macrophages from vitamin E-treated rats. *International Journal of Vitamin and Nutrition Research*, **60**, 338–342.
13. Sakamoto, W., Fujie, K., Mino, M. and Murota, S. (1993) Vitamin E and macrophage. *In*: M. Mino, Nakamura, H., Diplock, A.T. and Kayden, H.J. (eds): *Vitamin E- its usefulness in Health and in curing Diseases*. Japan Scientific Societies Press, Tokyo, pp209–217.
14. Heyworth, P., Karnovsky, M.L. and Badwey, J.A. (1989) Protein phosphorylation associated with synergistic stimulation of neutrophiles. *Journal of Biological Chemistry* **264**, 14935–14939.

15. Ambruso, D.R., Bolsher, B.G.J.M., Stokman, P.M., Verhoeven, A.J. and Roos, D. (1990) Assembly and activation of NADPH:O_2 oxidoreductase in human neutrophils after stimulation with phorbol myristate acetate. *Journal of Biological Chemistry*, **265**, 923–930.
16. Utsumi, T., Klostergaard, J., Akimaru, K., Edashige, K., Sato, E.F. and Utsumi, K. (1992) Modulation of TNF-α-priming and stimulation dependent superoxide generation in human neutrophils by protein kinase inhibitors. *Archives of Biochemistry and Biophysics*, **294**, 271–278.
17. Badwey, J.A., Robinson, J.M., Heyworth, P.G. and Curnutte, J.T. (1989) 1,2-dioctanoyl-*sn*-glycerol can stimulate neutrophils by different mechanisms. Evidence for a pathway that does not involve phosphorylation of the 47-kDa protein. *Journal of Biological Chemistry*, **264**, 20676–20682.
18. Burnham, D.N., Uhlinger, D.J. and Lambeth, J.D. (1990) Diacylglycerol synergizes with anion amphiphile to activate superoxide generation and phosphorylation of $p47^{phox}$ in cell-free system from human neutrophils. *Journal of Biological Chemistry*, **265**,
19. Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Ito, N., Shibuya, M. and Fukami, Y. (1987) Genistein, a specific inhibitor of tyrosine-specific protein kinases. *Journal of Biological Chemistry*, **262**, 5592–5595.
20. Miyahara, M., Watanabe, S., Okimasu, E. and Utsumi, K. (1987) Charge-dependent regulation of NADPH oxidase activity in guinea-pig polymorphonuclear leukocytes. *Biochimica et Biophysica Acta*, **929**, 253–262.
21. Shpungin, S., Dotan, I., Abo, A. and Pick, E. (1989) Activation of the superoxide forming NADPH oxidase in a cell-free system by sodium dodecylsulfate. *Journal of Biological Chemistry*, **264**, 9195–9203.
22. Takahashi, R., Edashige, K., Sato, E.F., Inoue, M., Matsuno, T. and Utsumi, K. (1991) Luminol chemiluminescence and active oxygen generation by activated neutrophils. *Archives of Biochemistry and Biophysics*, **285**, 325–330.
23. Yamamoto, M., Saeki, K. and Utsumi, K. (1991) Isolation of human salivary polymorphonuclear leukocytes and their stimulation-coupled responses. *Archives of Biochemistry and Biophysics*, **289**, 76–82.
24. Gelder, V.B.F. and Slater, E.C. (1962) The extinction coefficient of cytochrome c. *Biochimica et Biophysica Acta*, **58**, 593–595.
25. Edashige, K. Sato, E.F., Akimaru, K, Kasai, M. and Utsumi, K. (1992) Differentiation of HL-60 cells by phorbol ester is correlated with up-regulation of protein kinase C-α. *Archives of Biochemistry and Biophysics*, **299**, 200–205.
26. Tamai, H., Manago, M., Yokota, K., Kitagawa, M. and Mino, M. (1988) Determination of α-tocopherol in buccal mucosal cells using an electrochemical detection. *International Journal of Vitamin and Nutrition Research*, **58**, 202–207.
27. Cox, J.A., Jeng, A.Y., Blumberg, P.M. and Tauber, A.I. (1987) Comparison of subcellular activation of the human neutrophil NADPH-oxidase by arachidonic acid, sodium dodecyl sulfate (SDS), and phorbol myristate acetate (PMA). *Journal of Immunology*, **138**, 1884–1888.
28. Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986) Staurosporine, a potent inhibitor of phospholipid/Ca^{2+}-dependent protein kinase. *Biochemical and Biophysical Research Communications*, **135**, 397–402.
29. Caldwell, S.E., McCall, C.E., Hendricks, C.L., Leone, P.A., Bass, D.A. and McPhail, L.C. (1988) Coregulation of NADPH oxidase activation and phosphorylation of a 48-kDa protein(s) by a cytosolic factor defective in autosomal recessive chronic granulomatous diseases. *Journal of Clinical Investigation*, **81**, 1485–1496.
30. Rotrosen D. and Leto TL, (1990) Phosphorylation of neutrophil 47-kDa cytosolic oxidase factor. *Journal of Biological Chemistry*, **265**, 19910–19915.

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