Regulation of Protein Kinase C-Dependent Superoxide Generation by α-Tocopherol in Neutrophils

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Key words : α-tocopherol, guinea pig neutrophil, protein kinase C, protein phosphorylation, stimulation-induced superoxide generation

Abstract

Various ligands activate leukocytes and generate superoxide radicals ($O_2^{\cdot-}$) via different pathways. Although α-tocopherol inhibits protein kinase C (PKC) *in vitro* and affects $O_2^{\cdot-}$ generation of phagocytic cells *in vivo*, the role of the vitamin in $O_2^{\cdot-}$ generation is not fully understood. Phorbol myristate acetate (PMA)-induced PKC-dependent $O_2^{\cdot-}$ generation in guinea pig peritoneal neutrophils (GPtPMN) *in vitro* was inhibited in concentration dependent manner by α-tocopherol. The ID_{50} was 1 μM for a 2 min incubation of GPtPMN (10^6 cells/ml) suspended in medium. Phosphorylation of 47 kDa protein stimulated by PMA was inhibited by α-tocopherol. Similar inhibition by α-tocopherol was observed in $O_2^{\cdot-}$ generation induced by dioctanoyl glycerol (DOG) and calcium ionophre (A23187)-induced $O_2^{\cdot-}$ generation but a little in formyl-methionyl-leucyl-phenylalanine (FMLP), opsonized zymosan (OZ) and sodium dodecyl sulfate (SDS)-induced $O_2^{\cdot-}$ generation. The inhibitory action of α-tocopherol decreased with the time of incubation with rat peritoneal neutrophils (RPMN) despite an increase in its intracellular concentration. Furthermore, the rate of $O_2^{\cdot-}$ generation was slightly higher in RPMN obtained from α-tocopherol-deficient rats but was not different in RPMN from α-tocopherol-supplemented rats. These results indicate that part of the PKC-dependent $O_2^{\cdot-}$ generation *in vitro* might be regulated by cellular levels of α-tocopherol although it is not regulated solely by α-tocopherol content.

Abbreviations used

Cyt. c, ferricytochrome C; DOG, L-α-1, 2-dioctanoyl glycerol (diC_8); DG, diacyl glycerol; FMLP, formyl-methionyl-leucyl-phenylalanine; GPtPMN, guinea pig peritoneal neutrophils; KRP, Krebs-Ringer phosphate; $O_2^{\cdot-}$, superoxide anion; OZ, opsonized zymosan; PKC, protein kinase C or Ca^{2+} and phospholipid dependent protein kinase; PMA, phorbol 12-myristate 13-acetate; RPMN, rat peritoneal neutrophils; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

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Introduction

It is well known that α-tocopherol (vitamin E) acts as an antioxidant[1~3]. Recently, Mahoney and Azzi[4,5] found that α-tocoherol inhibits brain PKC *in vitro*. This inhibition was concentration dependent, and half maximum inhibition occurred at a concentration of 30 μM. The inhibitory action of α-tocopherol on PKC was observed *in vivo* and found to parallel to the inhibition of smooth muscle cell proliferation by the vitamin[6~8]. The vitamin also inhibited phorbol ester-induced PKC translocation from the cytosol to the membrane which suggests a possible mechanism of PKC inhibition by α-tocopherol[9]. This inhibitory action was further confirmed an *in vivo* model system using the OZ-induced chemiluminescence of a cypridina luciferin analogue in polymorphonuclear cells[10]. Similar suppression of superoxide generation by α-toco-

pherol was observed in PMA-stimulated macrophages isolated from rats administered α-tocopherol orally[11,12]. Recently, it has been postulated that there are more than two pathways of $O_2^{\cdot-}$ generation in neutrophils[13~19], one of which is a PKC-mediated reaction induced by PMA or diacyl glycerol (DG) and/or by calcium ionophore in which the $O_2^{\cdot-}$ generation was strongly inhibited by PKC inhibitors[13~15]. The other one is a receptor-mediated reaction induced by FMLP or OZ, which is inhibited by tyrosine kinase inhibitors[15~18]. Another reaction is a modulation of the physicochemical nature of the membrane by sodium dodecyl sulfate (SDS) or arachidonate[18,19]. Thus the complete molecular picture of the role of the inhibitory action of α-tocopherol against neutrophil $O_2^{\cdot-}$ generation is not fully understood. In the present study, we examined the ability of α-tocopherol to inhibit PKC-media-ted processes in intact cells using the PMA-or DG-stimulated respiratory burst of neutrophils as a model system, comparing it to its action against other $O_2^{\cdot-}$ generating reactions induced by various stimuli such as FMLP, arachidonate, Ca^{2+} ionophore (A23187) and SDS. We found that the PKC-dependent $O_2^{\cdot-}$ generation of neutrophils is strongly inhibited by α-tocopherol *in vitro* and that most α-tocopherol distributed throughout cells is not able to inhibit PKC-dependent $O_2^{\cdot-}$ generation.

Materials and Methods

Chemicals

A23187, ferricytochrome c (Cyt. c), FMLP, PMA, sodium arachidonate, staurosporine and zymosan were purchased from Sigma Co. (St. Louis, Mo). Dioctanoyl glycerol (diC_8) was purchased from Funakoshi Pharmaceutical Co. (Tokyo). Nutrose was obtained from Eastman Kodak Co. (Rochester, N.Y.). [^{32}P]orthophosphate were obtained from ICN Radiochemicals (Irvine, Ca). 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

GPtPMN and RPMN were isolated from peritoneal cavity of guinea pig and rat 16hr after intraperitoneal injection of 2% Nutrose and washed twice with calcium-free KRP, pH 7.4, as described by Takahashi *et al.* [20,21]. Neutrophils were stimulated by 1.25×10^{-8}M FMLP, $1-2\times10^{-9}$M PMA, 1×10^{-6}M DOG (diC_8), 1×10^{-4}M SDS, 200 μg/ml OZ and 10^{-6}M A23187 at 37°C.

Measurement of $O_2^{\cdot-}$ generation

$O_2^{\cdot-}$ generation 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[14,20]. Briefly, the reaction was started by adding neutrophils (1×10^6 cells/ml) at 37°C in the medium of KRP containing 10mM glucose, 100μM Cyt. c and 1mM $CaCl_2$ in the presence or absence of various ligands. The change in absorbance at 550-540nm ($A_{550-540}$)[22] was monitored continuously.

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 the 5% CO_2 and 95% air for 30min, and carrier free [^{32}P]orthophosphate (1mCi/ml) was added. After 1hr, the cells were washed with phosphate-free RPMI and the cells (2×10^6 cells/ml) were suspended in the same medium for 10min at 37°C. Thereafter, various concentrations of α-tocopherol were added and the reaction mixture was incubated for 2min at 37°C. One μl/ml of 10^{-6}M PMA was added and the mixture was incubated for another 3min. Final concentrations of α-tocopherol and PMA were 0.01 -10μM and 1nM, respectively. The reaction was terminated by adding ice-cold 45% trichloroacetic acid (TCA) solution containing 2mM phenylmethylsulfonyl fluoride to yield a final concenration of 15 % TCA. The precipitant was washed twice with ice-cold ether/ethanol (1:1) and dissolved in sodium dodecyl sulfate (SDS) sample buffer. SDS-poly-

acrylamide gel electrophoresis (SDS-PAGE) was carried out with 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[23].

Determination of α-tocopherol content in neutrophils

α-Tocopherol content in the cells was determined using HPLC with an electrochemical detector following the method of Tamai *et al*[24]. Briefly, one ml of cell suspension (1×10^6 cells) and 1ml of tocol in ethanol were suspended in centrifuge tube together with 1ml of 6% pyrogallol solution in ethanol and preincubated for 2min at 70°C. The incubation mixture was added to 0.2ml of 60% KOH and saponified at 70°C for 30min and then cooled with water and mixed with 2.5ml of distilled water and 5ml of n-hexane. The mixture was vigorously shaken for 5min and then centrifuged at 3,000rpm for 5min. The 4ml hexane layer was evaporated under nitrogen gas flow at 40°C and dissolved in 50μl of ethnol and analyzed by HPLC (Shimazu LC-6A) with the Shimazu electrochemical detector (L-ECD-6A) using 4.6×150mm 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

Stimulus specjfic $O_2^{\cdot-}$ generation of neutrophils and various inhibitors

As described previously, different pathways have been postulated for the stimulation-dependent $O_2^{\cdot-}$ generation of GPtPMN. Figure 1 shows $O_2^{\cdot-}$ generation by different stimuli and inhibition by different inhibitors. FMLP-or OZ-induced $O_2^{\cdot-}$ generation was inhibited by genistein[25], a tyrosine kinase inhibitor. The inhibited $O_2^{\cdot-}$ generation was then stimulated by PMA or DOG and the following $O_2^{\cdot-}$ generation was inhibited by staurosporine[26], an inhibitor of PKC. The $O_2^{\cdot-}$ generation was again induced by SDS and inhibited by cetylamine, an cationic amphiphile. The results show that the inhibitors of PKC do not inhibit FMLP-or SDS-induced $O_2^{\cdot-}$ generation and that genistein does not inhibit PMA-induced $O_2^{\cdot-}$ generation[15]. By contrast, cetylamine inhibited $O_2^{\cdot-}$ generation stimulated by any of the agents[18,27]. These results indicate that there are different pathways for stimulus-dependent $O_2^{\cdot-}$ generation. Figure 2 shows a schematic representation of the different pathways for stimulation of $O_2^{\cdot-}$ generation in neutrophils via NADPH oxidase, and the inhibitors of the pathways.

Effect of α-tocopherol on $O_2^{\cdot-}$ generation stimulated by different stimuli

Figure 3 shows the effect of α-tocopherol (1μM) on stimulation-dependent $O_2^{\cdot-}$ generation in GPtPMN by various stimuli *in vitro*. PKC-dependent $O_2^{\cdot-}$ generation induced by PMA-or DOG-and /or A23187 was inhibited by addition of α-tocopherol. However, receptor-and anionic amphiphile-induced

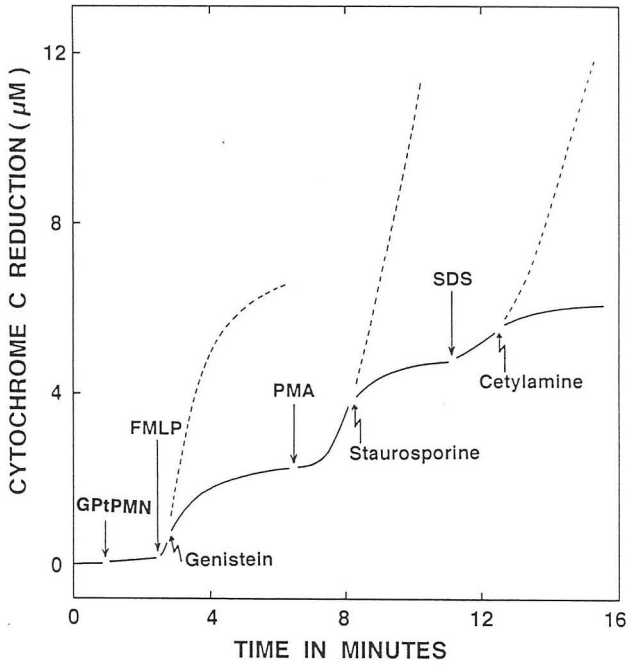

Fig. 1 $O_2^{\cdot-}$ generation of neutrophils by various stimuli and its inhibition
GPtPMN : 1×10^6 cells/ml FMLP : 1.25×10^{-8}M
PMA : $1-2\times10^{-9}$M SDS : 1×10^{-4}M

$O_2^{\cdot-}$ generation was not inhibited even at a concentration of 10 μM α-tocopherol.

Effect of various concentrations of α-tocopherol on PMA-dependent neutrophil $O_2^{\cdot-}$ generation of GPtPMN

Inhibition of neutrophil $O_2^{\cdot-}$ generation by α-tocopherol was concentration dependent and the ID_{50} was 1 μM after 2 min incubation with the vitamin (Fig. 4). The inhibitory pattern was quite similar to that of staurosporine, an inhibitor of PKC. Therefore, the effect seems to depend on the inhibitory action of α-tocopherol against PKC activity. However, the ID_{50} of α-tocopherol (1 μM) for the inhibition of $O_2^{\cdot-}$ generation was much lower than that for PKC inhibition (ID_{50}, 30 μM)[4,5]. The difference of ID_{50} between $O_2^{\cdot-}$ generation and PKC activity might be due to the accumulation of added α-tocopherol in the neutrophyl membrane or the sites of distribution of α-tocopherol which are inaccessible to PKC. Similar inhibitory effects of α-tocopherol were observed on human peripheral blood, human saliva, human cord blood and rat peritoneal neutrophils (data not shown).

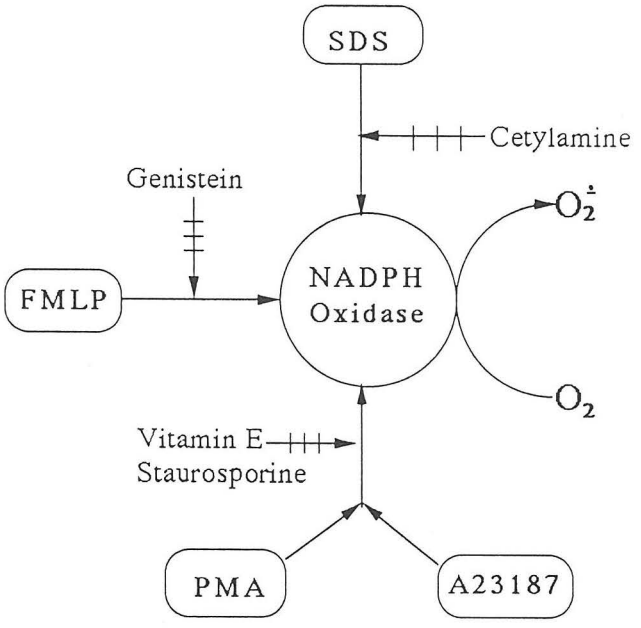

Fig. 2 A shematic representation of the different pathways for stimulation of $O_2^{\cdot-}$ generation via NADPH oxidase in neutrophils

Effect of α-tocopherol on the PMA-induced phosphorylation of GPtPMN 47 kDa protein

In the studies of patients with autosomal chronic granulomatous diseases, two cytoplasmic factors of

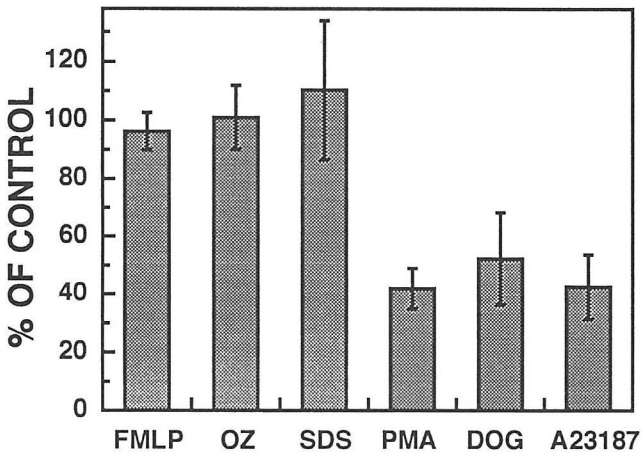

Fig. 3 Effect of α-tocopherol on $O_2^{\cdot-}$ generation stimulated by different stimuli
α-tocopherol : 1×10^{-6} M
OZ : 200 μg/ml
PMA : $1-2 \times 10^{-9}$ M
A23187 : 1×10^{-6} M
FMLP : 1.25×10^{-8} M
SDS : 1×10^{-4} M
DOG : 1×10^{-6} M

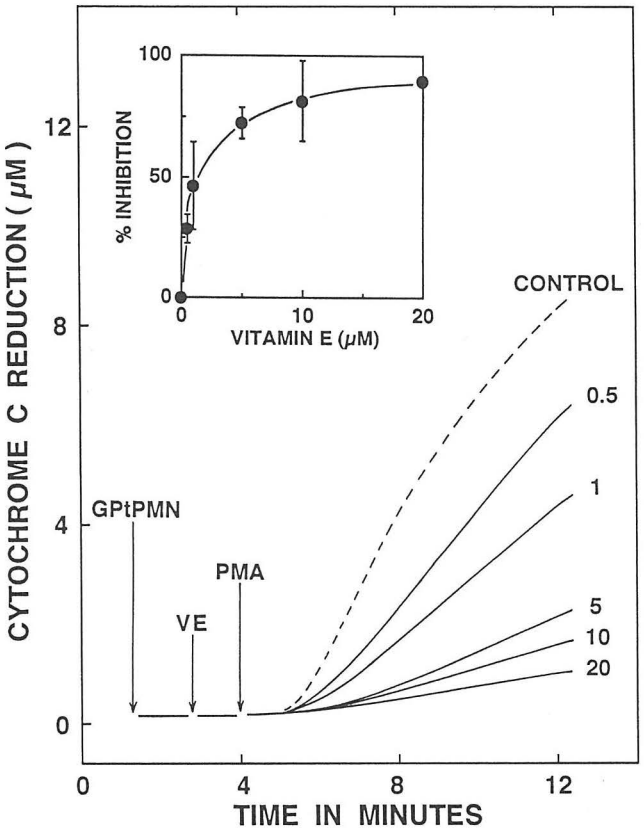

Fig. 4 Concentration dependency of α-tocopherol on PMA-induced $O_2^{\cdot-}$ generation

47 kDa ($p47^{phox}$) and 67 kDa ($p67^{phox}$) were found to be essential for $O_2^{\cdot-}$ generation in neutrophils[28,17], and the role of PKC in the phosphorylation of $p47^{phox}$ has been suggested to be important in the activation of NADPH oxidase[29]. To confirm the inhibition of PMA-induced phosphorylation of cytoplasmic protein by α-tocopherol, the effect of the vitamin on the incorporation of [^{32}P]orthophosphate into cytoplasmic $p47^{phox}$ of GPtPMN was examined. Many cytoplasmic proteins were phosphorylated without stimulation with PMA but phosphorylation of $p47^{phox}$ was enhanced by treatment with PMA. α-Tocopherol (1-10 μM) inhibited the phosphorylation of these neutrophil proteins including $p47^{phox}$ (Fig. 5). This result suggested that inhibition of phosphorylation of $p47^{phox}$ may be correlated with the inhibition of $O_2^{\cdot-}$ generation in neutrophils by α-tocopherol.

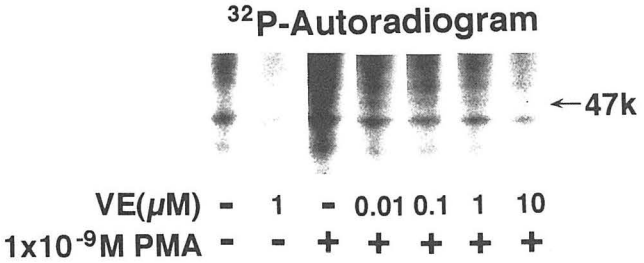

Fig. 5 Effect of α-tocopherol on the PMA-induced phosphorylation of 47 kDa protein in neutrophils

Changes in α-tocopherol content and PMA-induced $O_2^{\cdot-}$ generation of RPMN by addition of α-tocopherol in vitro

To obtain further insight into the mechanism of inhibition of $O_2^{\cdot-}$ generation by α-tocopherol, we investigated the relationship between α-tocopherol content and the inhibition of $O_2^{\cdot-}$ generation in RPMN by addition of α-tocopherol *in vitro*. No parallel relationship between the two parameters was observed. The content of α-tocopherol in normal RPMN was about 5-10 ng/10^6 cells/ml. This intracellular concentration was linearly increased by increasing addition of α-tocopherol, but the inhibitory activity was not linear (Fig. 6). The rate of PMA-induced $O_2^{\cdot-}$ generation was found to be very high at low concentrations of added α-tocopherol. Furthermore, the inhibitory action of α-tocopherol was dependent on the time of incubation with added α-tocopherol and decreased as the content of the vitamin increased (Fig. 7).

Relation between α-tocopherol content and $O_2^{\cdot-}$ generation in neutrophils obtained from α-tocopherol deficient and supplemented rats

To obtaine an insight into the mechanism of inhibition,the relationship between the α-tocopherol content of neutrophils and PMA-stimulated $O_2^{\cdot-}$ generation *in vivo* was investigated further. PMA-induced $O_2^{\cdot-}$ generation was measured in neutrophils

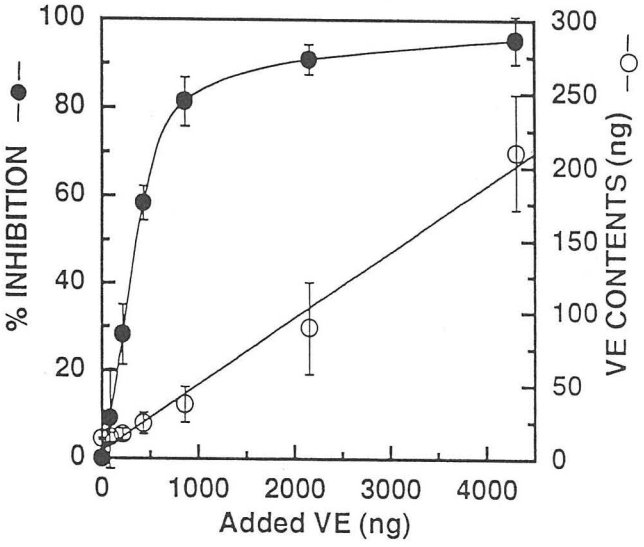

Fig. 6 α-Tocopherol content and PMA-induced $O_2^{\cdot-}$ generation in neutrophils

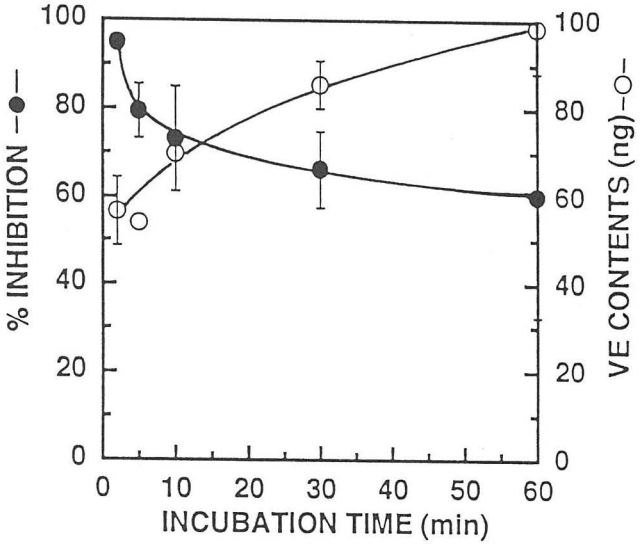

Fig. 7 Incubation time dependency of $O_2^{\cdot-}$ generatiom and α-tocopherol content

obtained from rats which had undergone different treatments either to supplement them with α-tocopherol or to make them α-tocopherol deficient. The PMA-induced $O_2^{\cdot-}$ generation in neutrophils obtained from α-tocopherol-deficient rats (0.53 n moles/10^6 cells/min) was slightly higher than that obtained from normal rats (0.42 n moles/10^6 cells/min) corresponding to the lower content of α-tocopherol (e.g. 2-5 ng/10^6 cells) (Table 1). On the contrary, the $O_2^{\cdot-}$ generation of neutrophils obtained from α-tocopherol supplemented rats showed no significant difference in their ability of PMA-induced $O_2^{\cdot-}$ generation instead of the increased content of α-tocopherol, such as 380 ng/10^6 cells (Table 1). The resuts indicate that only a small part of the α-tocopherol distributed in cells is able to regulate the $O_2^{\cdot-}$ generation.

Table 1 α-Tocopherol content and $O_2^{\cdot-}$ generation in neutrophils from α-tocopherol deficient and supplemented rats

	VE contens (ng/10^6 cells)	PMA induced superoxide generation (nmoles/10^6 cells/min)
Control	9.64 (±3.28)	0.42 (±0.151)
VE deficient (4 weeks)	2.27 (±1.012)	0.53 (±0.177)
VE supplemented (50 mg/kg, 3 days)	380.23 (±6.256)	0.45 (±0.222)

Conclusion

The results obtained in these experiments suggested that PMA-or DOG-induced $O_2^{\cdot-}$ generation in neutrophils cerresponded to the activiy of PKC and that the regulatory activity of α-tocopherol may operate via inhibition of PKC activity. However, the inhibitory effect was dependent on the time of incubation and prolonged incubation decreased the inhibitory activity. Furthermore, PMA-induced $O_2^{\cdot-}$ generation of RPMN obtained from α-tocopherol supplemented rats was not inhibited. These results suggest that α-tocopherol can regulate PKC-dependent signal transducing reactions of neutrophils under certain conditions but that the distribution of α-tocopherol in the membranes is changed with time of incubation until it reaches a situation where it can no longer affect the PKC-dependent $O_2^{\cdot-}$ generation of neutrophils. However, no direct evidence was obtained in these experiments to explain the inhibition of neutrophil $O_2^{\cdot-}$ generation by α-tocopherol through PKC mediated pathway. Futher experiments, therefore, are needed to understand the molecular mechanism of the inhibition of inhibited $O_2^{\cdot-}$ generation by added α-tocopherol.

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