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Inhibition of Neutrophil-Superoxide Generation by α-tocopherol and Coenzyme Q

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Effects of various derivatives of α-tocopherol (VE) and coenzyme Q (CoQ) on superoxide ($O_2^{\bullet-}$) generation of neutrophils and protein kinase C (PKC) activity were examined. VE and CoQ_8 inhibited $O_2^{\bullet-}$ generation of neutrophils stimulated by a protein kinase C mediated process monitored by cytochrome c reduction and spin trapping methods. The inhibitory action was observed not only with α-tocopherol, but also with β-, γ-, δ-tocopherols and with tocol which is a chemical similar to VE but lacking methyl groups on the chromanol ring structure and which is not a radical scavenger. By contrast, no inhibition was observed with 2-carboxy-2,5,7,8-tetramethyl-6-chromanol (CTMC, trolox) or 2,2,5,7,8,-pentamethyl-6-chromanol (PMC) which are water soluble VE derivatives having radical scavenging activity. Compounds having a similar isoprenoid chain, such as CoQ, also have inhibitory activity on PKC-dependent $O_2^{\bullet-}$ generation of neutrophils. The inhibitory activity of CoQ derivatives is dependent on the length of the unsaturated isoprenoid chain. CoQ derivatives having 16,24 and 32 carbon isoprenoid chains corresponding to CoQ4,6, and 8 inhibited $O_2^{\bullet-}$ generation but 4 and 40 carbon isoprenoid chains corresponding to CoQ2 and 10 had no inhibitory activity on $O_2^{\bullet-}$ generation. Alpha-tocopherol and CoQ inhibited PKC activity but the ID_{50} for $O_2^{\bullet-}$ generation and PKC activity was different for each compound. However, no direct relationship between VE content and $O_2^{\bullet-}$ generation of neutrophils was observed. These results suggest that isoprenoids of VE and CoQ participate in the inhibition of the NADPH oxidase activation system through modulation of the neutrophil membrane probably by the inhibition of PKC.

Key words: α-tocopherol, coenzyme Q, inhibition of protein kinase C, isoprenoid chains, inhibition of neutrophil $O_2^{\bullet-}$ generation, DMPO/•OOH signal

Abbreviations: CTMC, 2-carboxy-2,5,7,8-tetramethyl-6-chromanol (trolox); CoQ, coenzyme Q or ubiquinone; Cyt.c, ferricytochrome c; DETAPAC, diethylenetriaminepentaacetic acid; DMPO, 5,5'-dimethyl-1-pyrroline-1-oxide; DMPO/•OH, 2,2-dimethyl-5-hydroxyl-1-pyrrolidinyloxyl; DMPO/•OOH, 2,2-dimethyl-5-hydroperoxyl-1-pyrrolidinyloxyl; DMSO, dimethylsulfoxide; DOG, L-α-1,2-dioctanoyl glycerol (diC_8); DSF, desferrioxamine; EPR, electrone paramagnetic resonance; FMLP, formyl-methionyl-leucyl-phenylalanine; G1 peptide, Arg-Arg-Arg-Val-Thr-Ser-Ala-Ala-Arg-Arg-Ser; GPMN, guinea pig peritoneal neutrophils; KRP, Krebs-Ringer phosphate; PMC, 2,2,5,7,8,-pentamethyl-6-chromanol; $O_2^{\bullet-}$, superoxide anion; OZ, opsonized zymosan; PKC, protein kinase C or Ca^{2+} and phospholipid dependent protein kinase;

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PMA, phorbol 12-myristate 13-acetate; RPMN, rat peritoneal neutrophils; SDS, sodium dodecyl sulfate

INTRODUCTION

Although it is well known that VE is a strong antioxidant,[1–3] Mahoney and Azzi recently found that VE also inhibited PKC activity not only *in vitro* but also *in vivo*[4,5] and prevented smooth muscle cell proliferation in parallel with PKC inhibition.[6–8] As a possible mechanism of inhibition of PKC, it was suggested that PMA-induced translocation of cytoplasmic factors from the cytosol to the membrane was inhibited by VE.[9] This inhibitory action of VE was further confirmed in an *in vivo* model system using the OZ-induced chemiluminescence of human neutrophils[10] and PMA-stimulated macrophages isolated from rats administered with VE.[11,12] Several lines of evidence are accumulating on the mechanism of the respiratory burst of neutrophils and it has been reported that different pathways activate the membrane-bound NADPH oxidase leading to $O_2^{\bullet-}$ generation.[13–19] One pathway is PKC-dependent[13–15] and the other is non-PKC- dependent.[15–19] In a previous paper,[20] we showed that VE inhibited PKC-mediated $O_2^{\bullet-}$ generation in intact cells but not non-PKC-mediated $O_2^{\bullet-}$ generation induced by various stimuli such as formyl-methionyl-leucyl-phenylalanine (FMLP), arachidonate and SDS. We also found that VE inhibited *in vivo* phosphorylation of neutrophil proteins, such as $p47^{phox}$.[20] These results suggest that inhibition of the PKC-mediated $O_2^{\bullet-}$ burst by VE seems to be due to the inhibition of protein phosphorylation but not to the chemical structure of VE derivatives related to the radical scavenging activity. However, the ID_{50} values for VE on PKC and $O_2^{\bullet-}$ generation were different, and no direct correlation between intracellular VE content and the generation of $O_2^{\bullet-}$ in neutrophils was observed. Thus, the precise mechanism of inhibition is not fully understood. In this report we describe the relationship between the VE content and the ability to inhibit stimulation-coupled $O_2^{\bullet-}$ generation and between chemical structures of VE or CoQ and their inhibition of the respiratory burst of neutrophils.

MATERIALS AND METHODS

Chemicals

VE and CoQ derivatives were kindly donated by the Eisai Co. Ltd (Tokyo). Desferrioxamine (DSF) was obtained from Ciba-Geigy (Japan) Ltd (Tokyo). Diethylenetriaminepentaacetic acid (DETAPAC), ferricytochrome c (Cyt. c) and PKC were purchased from Sigma Co. (St. Louis, MO) and Promega Co. (Wisconsin, USA), respectively. 5,5'-dimethyl-1-pyrroline-1-oxide (DMPO) was obtained from Daiichi Chemical Co. Ltd (Tokyo) and used without further purification. The Non-Radioisotopic Protein Kinase Assay kit was purchased from MBL Co. (Nagoya, Japan). Leupeptin and all other chemicals were from Nacalai Tesque (Kyoto). PMA and VE were dissolved in ethanol and CoQ was dissolved in dimethylsulfoxide (DMSO). The final concentrations of ethanol and DMSO in the reaction mixture were less than 0.5% and control experiments were carried out in the presence of 0.5% ethanol.

Neutrophils

Rat peritoneal neutrophiles (RPMN) and guinea pig peritoneal neutrophils (GPMN) were isolated from the peritoneal cavities of animals 16 hr after the intraperitoneal injection of 2% Nutrose and washed twice with calcium-free KRP, pH 7.4, as described in previous papers.[20–22] Neutrophils were stimulated by $1-2\times10^{-9}$ M PMA, 1.25×10^{-8} M formylmethionyl-leucyl-phenylalanine (FMLP) and 3×10^{-5} M sodium arachidonate at 37°C.

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

$O_2^{\bullet-}$ production was assayed by reduction of Cyt. c as described previously using a dual beam spectrophotometer (Shimadzu UV-3000)

quipped with a water-jacketed cell holder and agnetic stirrer.[14,21] Briefly, the reaction was arted by adding neutrophils (1×10^6 cells/ml) at 7°C to KRP medium containing 10 mM glucose, 00 μM Cyt. c and 1 mM $CaCl_2$ in the presence or bsence of various ligands. The change in ab-orbance at 550–540 nm ($A_{550-540}$)[23] was monitored ontinuously.

Assay of PKC activity

PKC was purified from rat brain according to the method of Walton and Bertics.[24] The activity of PKC was assayed using a Non-Radioisotopic Protein Kinase Assay kit (MBL Co., Nagoya). The kit is based on an enzyme linked immunosorbent assay (ELISA) that utilizes a synthetic peptide and a monoclonal antibody which recognizes phosphorylated form of peptide.[25] Briefly, the reaction was carried out in a mixture (120 μl) containing 800 nM PMA and 80 μM VE or CoQ. PKC catalyzed the phosphorylation of G1 peptide (Arg-Arg-Arg-Val-Thr-Ser-Ala-Ala-Arg-Arg-Ser; residues 3–13 of porcine Glial fibrillary acidic protein)[26] coated on the microwells. Mouse monoclonal antibody YC-10 was produced against synthetic peptide, Arg-Arg-Arg-Val-Thr-phosphoSer-Ala-Ala-Arg-Arg-phosphoSer (phosphopeptide G1). The antibody is bound to phosphopeptide G1 and is subsequently detected with anti-mouse IgG conjugated to peroxidase. Peroxidase substrate is then added to the microwell and the intensity of the color is measured photometrically at 429 nm.

Spin trapping and electrone paramagnetic resonance (EPR) spectrometry for $O_2^{\bullet-}$ and hydroxyradical ($^{\bullet}OH$)

Neutrophils (5×10^6cells/ml) were incubated in Krebs-Ringer-phosphate pH 7.4 (KRP) containing various concentrations of VE or CoQ, 2 mM DETAPAC, 2.5 mM DSF and 94 mM DMPO. The reaction mixture was transferred to a flat quartz EPR cuvette (0.3 mm thick) which was fixed to the cavity of an EPR spectrometer (JEOL JES FE-1X with 100-kHz field modulation, X-band). The microwave power used was 8 mW, and the magnetic field was 336.0 ± 5.0 mT. Sweeping time was 1 min. The EPR spectra of 2,2-dimethyl-5-hydroperoxy-1-pyrroli-dinyloxyl (DMPO/•OOH) and 2,2-dimethyl-5-hydroxy-1-pyrrolidinyloxyl (DMPO/•OH) aduct were identified from hyperfine parameters.[27,28]

Determination of VE content in neutrophils

VE content in the cells was determined using HPLC with an electrochemical detector following the method of Tamai *et al.*[29] Briefly, one ml of cell suspension (1×10^6 cells) and 1 ml of tocol in ethanol were suspended in a centrifuge tube together with 1 ml of 6% pyrogallol solution in ethanol and pre-incubated for 2 min at 70°C. The incubation mixture was added to 0.2 ml of 60% KOH and saponified at 70°C for 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 Shimadzu electrochemical detector (L-ECD-6A) using a 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).

RESULTS AND DISCUSSION

Effect of VE on the stimulation coupled generation of $O_2^{\bullet-}$

GPMN generate $O_2^{\bullet-}$ when activated with various stimuli, such as PMA, FMLP, arachidonic acid and opsonized zymosan (OZ). As reported in a previous paper,[20] the PKC-dependent activation of NADPH oxidase, for example $O_2^{\bullet-}$ generation induced by diacylglycerol or PMA, was inhibited by VE. The ID_{50} of VE was 1 μM. A similar

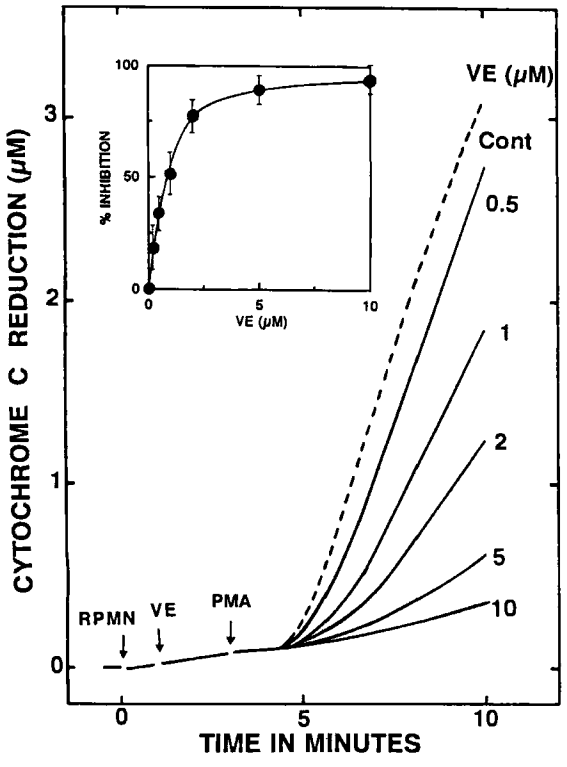

FIGURE 1 Effect of VE on the stimulation-dependent generation of $O_2^{\bullet-}$ from neutrophils. RPMN (10^6 cells/ml) were incubated in KRP (pH 7.4) containing 10 mM glucose and 1 mM $CaCl_2$ at 37°C. The total incubation volume was 2 ml and the respiratory burst was monitored spectrophotometrically by Cyt. c reduction. Numbers show the concentration of VE. The concentration of PMA was 10^{-9} M Data are mean +/− S.D. from 3 separate experiments. The % inhibition was measured 5 min after the addition of PMA.

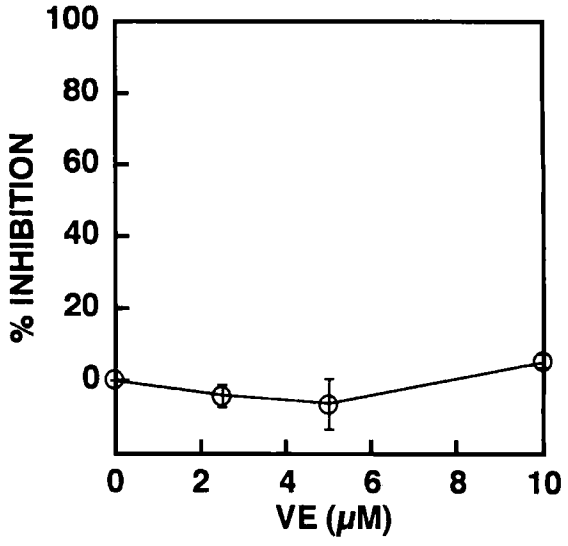

FIGURE 2 Effect of VE on the $O_2^{\bullet-}$ generation of RPMN induced by non-PKC dependent stimuli. Experimental conditions were as described in Figure 1 except that stimulation was by 3×10^{-5} M arachidonate. Data are mean +/− S.D. from 3 separate experiments.

concentration dependent inhibition of PMA-induced $O_2^{\bullet-}$ generation was observed with RPMN (Figure 1), but PKC-independent activation, such as arachidonate-induced $O_2^{\bullet-}$ generation,[18,19] was not inhibited by VE (Figure 2). These results showed that PMA- or DG-induced $O_2^{\bullet-}$ generation in neutrophils corresponded to the activation of PKC and that NADPH oxidase complex[30] is not a target of VE.

Effect of VE on the DMPO/•OOH signal of neutrophils stimulated with PMA

The inhibition of $O_2^{\bullet-}$ generation in PMA-stimulated GPMN by VE was investigated by EPR analysis of the generated active oxygen species. Figure 3 shows the EPR signal of neutrophils generated by stimulation by PMA.[27] The spectrum might be composed of DMPO/•OOH and DMPO/•OH because DMPO/•OOH is sensitive to SOD and DMPO/•OH originates from the internal conversion of DMPO/•OOH which takes place within 1–2 min. The EPR signal gradually increased just after addition of PMA to neutrophils and changed to the DMPO/•OH signal during the time after incubation with neutrophils in a flat quartz cell. The DMPO/•OOH signal was suppressed by VE (Figure 3). Thus, by means of the EPR signal of DMPO/•OOH, we confirmed that VE inhibited the generation of $O_2^{\bullet-}$ from PMA-stimulated neutrophils.

Effect of various VE derivatives on the $O_2^{\bullet-}$ generation of RPMN

To study the relationship between the chemical structure of VE and its inhibitory effect on $O_2^{\bullet-}$ generation of neutrophils, the effects of various

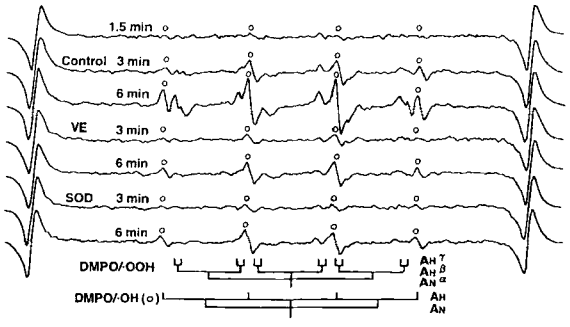

FIGURE 3 Effect of VE on the EPR signal of DMPO/•OOH and DMPO/•OH generated by GPMN stimulated by PMA. EPR spectra of DMPO/•OOH or DMPO/•OH generated from neutrophils stimulated by 10^{-7} M PMA were monitored in KRP medium containing 5×10^6 cells/ml, 94 mM DMPO, 2.3 mM DSF and 2 mM DETAPAC in the presence or absence of various concentrations of VE. The spectra were recorded from 1.5 min to 6 min after incubation with stimuli. Closed circles indicate DMPO/•OH signal and bars indicate DMPO/•OOH signal after stimulation with PMA in the presence or absence of VE (10 μM) and SOD (100 U/ml). The hyperfine splitting for DMPO/•OOH was $A_N\alpha$ = 14.3G, $A_H\beta$ = 11.7G, and $A_H\gamma$ = 1.25G.

VE derivatives on the PMA-stimulated $O_2^{\bullet-}$ generation of RPMN was examined. In common with α-tocopherol, $O_2^{\bullet-}$ generation of neutrophils was inhibited by various tocopherol derivatives, such as the β-, γ-, δ-tocopherols, which have radical scavenging activity[3] (Table 1). Moreover, tocol, a substance with the same side chain as α-tocopherol and which is inactive as a vitamin but scavenges radicals, showed a similar inhibitory activity against $O_2^{\bullet-}$ generation by PMA-stimulated neutrophils. On the contrary, no inhibition of the $O_2^{\bullet-}$ generation was observed on treatment with water soluble tocopherol derivatives, such as CTMC and PMC having radical scavenging activity,[31,32] at a concentration of 1 μM. These results indicate that the isoprenoid side chain has an important role in the inhibition of PKC-dependent $O_2^{\bullet-}$ generation but that no relationships exist between the radical scavenging activity of tocopherols and their derivatives and their inhibitory activity on $O_2^{\bullet-}$ generation of neutrophils.

TABLE 1 Effect of various derivatives of VE on the respiratory burst of RPMN neutrophils by PMA.

	PMA induced superoxide generation (% of control)
α-Tocopherol	31.92 ± 7.00
β-Tocopherol	36.03 ± 0.14
γ-Tocopherol	29.53 ± 3.63
δ-Tocopherol	29.99 ± 3.65
Tocol	34.03 ± 4.91
PMC	96.04 ± 3.80
Trolox	94.36 ± 7.48
Phytol	65.84 ± 6.75

Experimental conditions were the same as described in Figure 1. Data are mean +/− S.D. from 3 separate experiments.

Effect of CoQ_4 and its derivatives on PMA-stimulated $O_2^{\bullet-}$ generation of GPMN

As CoQ has a similar side chain to VE,[33] we tested the effect of CoQ_4 on PMA-induced $O_2^{\bullet-}$ generation of RPMN. Figure 4 shows the concentration dependent inhibition of PMA-stimulated $O_2^{\bullet-}$

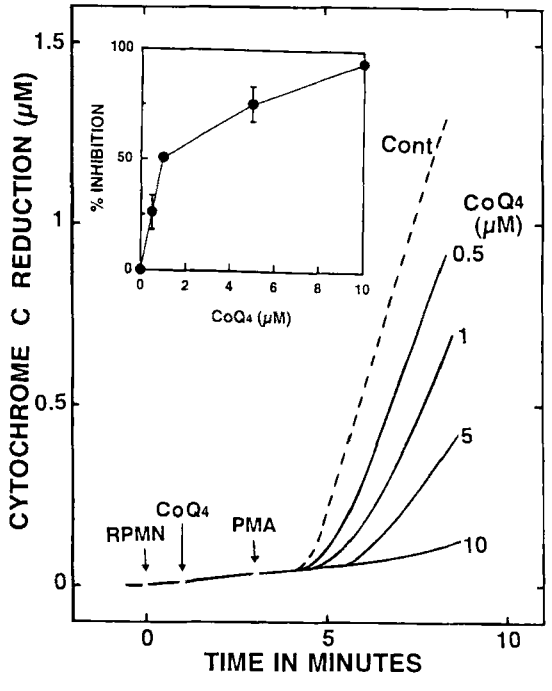

FIGURE 4 Concentration-dependent inhibition of PMA-induced $O_2^{\bullet-}$ generation of RPMN by CoQ_4. Experimental conditions were the same as described in Figure 1 except that various concentrations of CoQ_4 were added to inhibit $O_2^{\bullet-}$ generation. Data are mean +/− S.D. from 3 separate experiments.

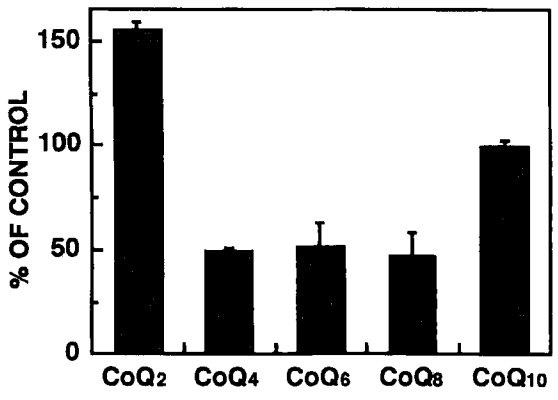

FIGURE 5 Effect of CoQ_4 on the $O_2^{\bullet-}$ generation of GPMN induced by various stimuli. Experimental conditions were in Figure 1 except that the number of neutrophils used and the concentration of CoQ were 10^6 GPMN and 1 μM, respectively. Data are mean +/− S.D. from 3 separate experiments.

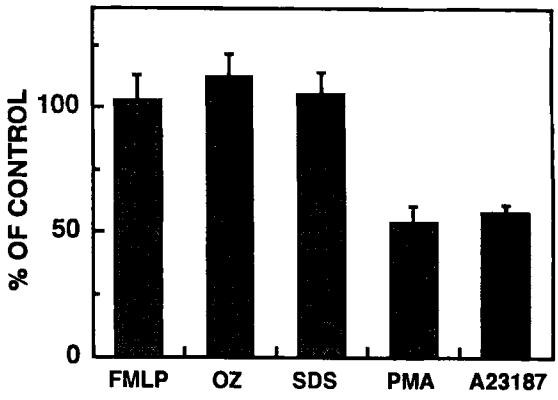

FIGURE 6 Effect of various CoQ derivatives on PMA-induced $O_2^{\bullet-}$ generation of RPMN. Experimental conditions were as described in Figure 1 except that 1 μM of various CoQs was added to separate incubations before the addition of PMA. Data are mean +/− S.D. from 3 separate experiments.

generation of neutrophils by CoQ_4. The ID_{50} was 1 μM. This concentration was quite close to the ID_{50} of VE.[16–19] CoQ_4 also inhibited DMPO/•OOH signal of neutrophils stimulated by a protein kinase C mediated process (data not shown). Furthermore, the inhibition was observed only with PKC-dependent $O_2^{\bullet-}$ generation and not with PKC-independent $O_2^{\bullet-}$ generation, such as arachidonate- or OZ-induced reactions, as observed with VE (data not shown). Since a large amount of $O_2^{\bullet-}$ was generated in casein-induced guinea pig peritoneal neutrophils (GPMN) when induced by various stimuli, we tested the effect of CoQ_4 on $O_2^{\bullet-}$ generation of GPMN. Figure 5 shows $O_2^{\bullet-}$ generation induced by PKC-dependent processes but not by PKC-independent processes was inhibited by CoQ_4. These inhibitory effects of CoQ derivatives on PMA-induced $O_2^{\bullet-}$ generation of RPMN were different for each CoQ derivative. CoQ_8, CoQ_6 and CoQ_4 inhibited the $O_2^{\bullet-}$ generation but not CoQ_{10} or CoQ_2 as shown in Figure 6. The mechanisms of the different inhibitory activities of these various CoQs is not known. However, it is thought that one factor which may be responsible for the difference in inhibitory activity of various CoQs is the difference in their incorporation into the plasma membrane where PKC is distributed.[34]

Effect of incubation time with VE on the inhibition of PMA-induced $O_2^{\bullet-}$ generation of neutrophils by VE

In the course of analysis of the inhibition mechanism, we found that the rate of inhibition by VE was decreased concomitant with an increase in the amount of VE incorporated during the time of incubation as shown in Figure 7. However, the

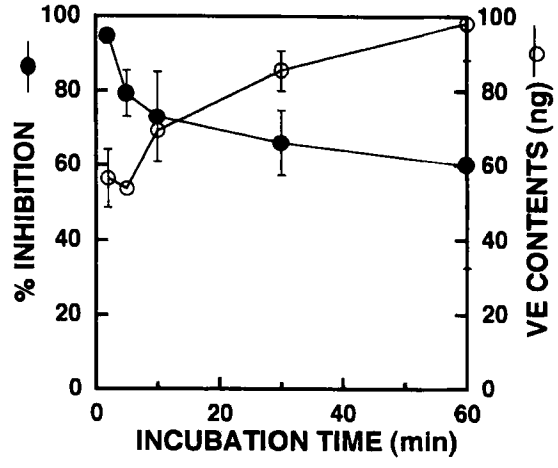

FIGURE 7 Effect of incubation time on the inhibitory activity of VE against PMA-induced $O_2^{\bullet-}$ generation of neutrophils and on the incorporation of VE by neutrophils. Experimental conditions were as described in Figure 1. Data are mean +/− S.D. from 3 separate experiments.

inhibitory activity of VE relative to the VE incorporated into RPMN during the time of incubation after addition of VE to the PMN suspension was not linearly proportional. In this context, the incorporation is dependent on the VE binding protein.[35] These results indicate that the distribution of VE after incorporation and accumulation in neutrophils might be changed and this change made VE to become inaccessible to PKC.

Relationship between the content of VE and PMA-stimulated $O_2^{\bullet-}$ generation of RPMN

To obtain further insight into the mechanism of inhibition by VE of neutrophil $O_2^{\bullet-}$ generation, the relationship between VE content and the activity of PMA-induced $O_2^{\bullet-}$ generation of RPMN was studied. To obtain neutrophils containing different amounts of VE, rats were fed with a vitamin E deficient diet for 4 weeks or administered with vitamin E (5 mg/rat) for 5 to 6 days. Both neutrophils obtained from VE deficient rats and rats administered with VE took up VE from the blood by means of the VE binding protein[35] and the VE contents was significantly different between the two types of neutrophils. However, the rate of PMA- stimulated $O_2^{\bullet-}$ generation of neutrophils obtained from VE deficient rats was only slightly higher than that of control neutrophils. Furthermore, the rate of $O_2^{\bullet-}$ generation in RPMN obtained from rats administered with VE was slightly less than that of untreated control rats (Table 2). The differences in $O_2^{\bullet-}$ generating activity between these two types of neutrophils were not significant. These results indicate that the incorporated VE is unable to inhibit PKC activity as observed in neutrophils just after treatment with VE.

Effect of α-tocopherol and CoQ on the protein kinase C activity

To obtain further insight into the mechanism of inhibition of neutrophil $O_2^{\bullet-}$ generation by α-tocopherol and CoQ, the effect of these reagents on PKC activity was examined. PKC activity was inhibited both by α-tocopherol and CoQ. The inhibitory activities of VE derivatives were quite similar to those for α-tocopherol and CoQ. Alpha-, β-, γ-, and δ-tocopherol and tocol each inhibited PKC but PMC and trolox did not inhibit PKC activity (Figure 8). Furthermore, PKC activity was inhibited by CoQ_4, CoQ_6 and CoQ_8 but not by CoQ_2 and CoQ_{10} as observed for the inhibition of neutrophil $O_2^{\bullet-}$ generation by these CoQs (Figure 8).

In these experiments we confirmed that inhibition of PMA-stimulated $O_2^{\bullet-}$ generation and DMPO/•OOH signal of neutrophils by VE depends on its isoprenoid side chain and not on its radical scavenging activity. Similar inhibition of $O_2^{\bullet-}$ generation and DMPO/•OOH signal was also observed with CoQ. Furthermore, the length of side chain is also important for the inhibitory activity of CoQ on PMA-induced $O_2^{\bullet-}$ generation of neutrophils. It was also found that the distribution of VE after incorporation into neutrophils changed from the cell membrane to other intracellular membranes where VE does not appear to be available for inhibition of some enzymes which activate the respiratory burst, such as PKC. Therefore, further studies are needed to clarify the molecular mechanism of inhibition of $O_2^{\bullet-}$

TABLE 2 Relationship between VE content and PMA-induced respiratory burst activity of neutrophils obtained from different sources.

	VE contents (ng/10^6cells)	PMA induced superoxide generation (nmoles/10^6cells/min)
Control	9.64 ± 3.28	0.42 ± 0.15
VE deficient (4weeks)	2.27 ± 1.01	0.53 ± 0.18
VE supplemented (50mg/kg,3days)	380.23 ± 6.26	0.45 ± 0.22

Experimental conditions were as described in Figure 1 except that neutrophils were obtained from normal, 4 weeks VE-deficient and 3 days VE-supplemented rats. Data are means +/− S.D. from 5 separate experiments.

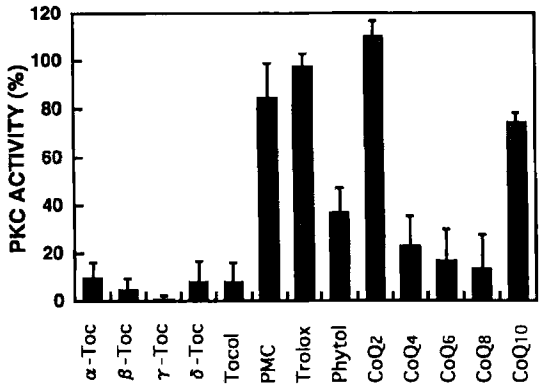

FIGURE 8 Effect of VE and CoQ on protein kinase C activity. The activity of PKC was assayed using a Non-Radioisotopic Protein Kinase Assay kit (MBL Co., Nagoya). The incubation was carried out in a mixture (120 μl) containing 8×10^{-9} M PMA, GI peptide and 80 μM VE or CoQ and phosphorylated GI peptide was detected by mouse monoclonal antibody (YC-10) of the phospho-peptide. The bound antibody was detected with anti-mouse IgG conjugated to peroxidase and the color intensity was measured photometrically at 429 nm in the presence of peroxidase substrate. Concentration of VE and CoQ was 80 μM. Data are mean +/− S.D. from 3 separate experiments.

generation of neutrophils by VE and its biological role in neutrophil activation.

Acknowledgements

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