Novel Water-Soluble Derivatives of Docosahexaenoic Acid Increase Diacylglycerol Production Mediated by Phosphatidylcholine-Specific Phospholipase C (43592)

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Abstract. **The effect of ascorbic acid 6-docosahexaenoate (DHA-VC) on the phospholipase-C-mediated hydrolysis of phosphatidylcholine was investigated. In human non-small cell lung cancer cells (PC-14) exposed to DHA-VC for 24 hr, a dose-dependent increase in phosphatidylcholine-specific phospholipase C (PC-PLC) activity was seen. PC-PLC activity in whole-cell homogenate of PC-14 cells was increased about 2.5-fold by 2 hr of treatment with DHA-VC (20 μg/ml). Treatment with DHA-VC also augmented PC-PLC activity in the crude membrane extract. On the other hand, DHA-VC inhibited the activity of phospholipase A_2 (ID_{50} = 800 μg/ml). Another water-soluble analog, choline docosahexaenoate, also stimulated PC-PLC activity. To explore the effect of DHA-VC on phosphatidylcholine turnover, we analyzed phospholipids labeled with [^{14}C] choline or [^{3}H]myristate by thin-layer chromatography, and found that the amount of [^{14}C]- and [^{3}H]-labeled phosphatidylcholine was constant in the presence of DHA-VC. These results suggest that phosphatidylcholine turnover was not influenced by DHA-VC. DHA-VC treatment increased protein kinase C activity of the cells in the late phase (120 min), suggesting that DHA-VC-induced diacylglycerol production mediated by PC-PLC causes protein kinase C activation. Considering that significant inhibition of DNA synthesis occurred 12 hr after 2 hr of treatment with DHA-VC (20 μg/ml), DHA-VC-induced PC-PLC activation seems to be an early event in DHA-VC-induced cytotoxicity, which suggests that the effects of DHA-VC on signal transduction pathways may play an important role in the cytotoxicity of DHA-VC.** [P.S.E.B.M. 1993, Vol 203]

Docosahexaenoic acid is an ω3 polyunsaturated fatty acid with growth-inhibitory effects on several tumors (1–3).

In recent years, investigators have started to focus attention on fish oils, which may influence the malignant process (1). The beneficial effects of fish oils against cancer have been attributed to their n-3 fatty acid content (e.g., eicosapentaenoic acid and docosahexaenoic acid), which affects the levels of prostaglandin E_2 (1, 4, 5). However, it has been unclear whether the n-3 fatty acid acts on phospholipase A_2, which produces prostaglandin E_2 from phosphatidylcholine, and whether the effect of these unsaturated fatty acids on phospholipase A_2 is critical for tumor growth inhibition. We examined the effect of docosahexaenoic acid analogs on phospholipid metabolism, focusing especially on phosphatidylcholine metabolism mediated by phosphatidylcholine-specific phospholipase C (PC-PLC). This pathway is one of the sources of diacylglycerol (DAG), which acts as a second messenger in the signal transduction pathway and has been shown to play an important role in tumor cell growth.

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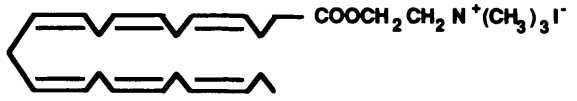
We have synthesized two new water-soluble analogs of docosahexaenoic acid, ascorbic acid 6-docosahexaenoate (DHA-VC) and choline docosahexaenoate (DHA-cho) (Fig. 1). In the present study, we have investigated the effect of these water-soluble analogs on the phosphatidylcholine hydrolysis pathway, and demonstrated that the compounds increased PC-PLC activity and DAG production.

Materials and Methods

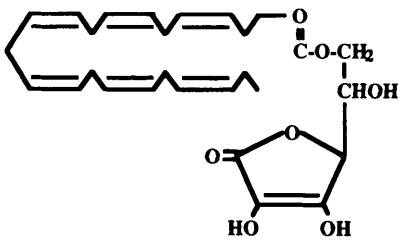
Chemicals. RPMI 1640 and calcium- and magnesium-free Dulbecco's phosphate-buffered saline (PBS) were purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). Phosphatidylcholine, 1-stearoyl-2-[*methyl*-^{14}C]arachidonyl ([^{14}C]phosphatidylcholine), [^{14}C]choline, and [^{3}H]myristate were purchased from Amersham Japan (Tokyo, Japan). Other drugs and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) if not otherwise mentioned.

Cell Cultures. PC-14, PC-9, and PC-7 are human non-small cell lung cancer cell lines (6). H69 is a small cell lung cancer cell line (7). Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) in a humidified 5% CO_2 atmosphere at 37°C.

Growth-Inhibition Assay. To elucidate the growth-inhibitory effects of drugs, we used the tetrazolium dye assay of Mossmann (8). Two hundred microliters of an exponentially growing cell suspension (5×10^4 cells/ml) were seeded in each well of a 96-well microtiter plate and incubated for 12 hr. The optical density was then measured at 562 and 630 nm using a Bio-Tek microplate reader (EL-340; Bio Metallics, Princeton, NJ). Each experiment was performed in triplicate and carried out more than three times independently. The 50% inhibitory dose (IC_{50}) was defined as the dose needed for 50% reduction of optical density in each test, and was calculated as (mean absorbance in 3 wells containing drugs − absorbance in 3 control wells)/(mean absorbance in 3 drug-free wells − absorbance in 3 control wells) × 100. Relative resistance was defined as IC_{50} for resistant subline/IC_{50} for parental cell line.



Choline docosahexaenoate (DHA-cho)



Ascorbic acid 6-docosahexaenoate (DHA-VC)

Figure 1. Structures of DHA-cho and DHA-VC.

Analysis of PC-PLC and Phospholipase A_2 Activities in DHA-VC- or DHA-cho-Treated Cells. PC-14 cells were treated with various doses of DHA-VC and DHA-cho for 2 hr. The cells were then harvested and membrane extraction was performed according to the methods described in Preparation of Membrane Fraction (see below). The activities of PC-PLC and phospholipase A_2 in the extracts were analyzed by the methods described in Analysis of Phosphatidylcholine Hydrolysis (see below).

Preparation of Membrane Fraction. Subconfluent cells were harvested and washed twice with ice-cold Buffer 1 (PBS containing 1 m*M* EDTA, pH 7.3). The collected cells were resuspended in Buffer 2 (2 m*M* HEPES, 154 m*M* NaCl, and 1 m*M* EDTA, pH 7.4) at 6×10^6 cells/ml. After freezing and thawing twice, the cell suspension was sonicated for 30 sec. Before ultracentrifugation, an aliquot of fresh sonicate was centrifuged at 180*g* for 10 min at 4°C. The supernatant was then centrifuged at 100,000*g* for 90 min at 4°C in an TL-100 ultracentrifuge (Beckman) with a TL-45 fixed-angle rotor (Beckman). After ultracentrifugation, the pellet was resuspended in Buffer 2. Membrane fractions were immediately frozen at −80°C until use. Protein content was measured by the method of Lowry *et al.* (9).

Analysis of Phosphatidylcholine Hydrolysis. [^{14}C]Phosphatidylcholine (sp act 56 mCi/mmol) was dried under N_2 gas and stored in chloroform at −20°C until use. At the time of the experiments, 22.4 μCi of [^{14}C]phosphatidylcholine from the stock solution was suspended in 100 μl of dH_2O and sonicated for 2 min at room temperature. Fifty micrograms of membrane fraction protein were incubated with 100 μl each of reaction buffer, [^{14}C]phosphatidylcholine solution, and 6 m*M* $CaCl_2$ for 1 hr at 37°C. The reaction buffer consisted of 100 m*M* HEPES and 100 m*M* sodium acetate (pH 7.5). Parallel reactions, in which 5 units of phospholipase A_2 or 10 units of phospholipase C (Seikagaku Kogyo, Co., Tokyo, Japan) were included instead of membrane fraction proteins, served as control experiments. Reactions were terminated by the addition of 1 ml of chloroform/methanol (2:1) (v/v), which contained 36 m*M* HCl. All measurements were performed in triplicate. A nonradiolabeled lipid mixture (60 nmol each of phosphatidylcholine, lysophosphatidylcholine, oleic acid, DAG, and sphingomyelin) was added just before lipid extraction for visualization of phosphatidylcholine, lysophosphatidylcholine, and DAG on thin-layer chromatography plates. The solutions were mixed and incubated for 1 hr at 4°C.

After incubation, phase separation was facilitated by centrifugation at 200*g* for 5 min. The chloroform

phase was transferred to a new glass tube. The residual aqueous phase was extracted again with 0.8 ml of chloroform and combined with the former chloroform phase. The pooled chloroform phases were dried under N_2 gas, dissolved in 20 μl of chloroform/methanol (2:1) (v/v) and then applied to the silica gel F254. For the fractionation of [^{14}C]DAG, diethyl ether/benzene/ethanol/triethylamine (40:50:2:1) (v/v/v/v) was used as the first-dimension solvent system.

Chloroform/methanol/acetic acid (85:14:1) (v/v/v) was used as the second-dimension solvent system. For the fractionation of [^{14}C]lysophosphatidylcholine and [^{14}C]phosphatidic acid, the plates were developed in chloroform/methanol/concentrated ammonia (65:35:5) (v/v/v). Lysophosphatidylcholine (Rf = 0.10), phosphatidic acid (Rf = 0.05), and sphyngomyelin (Rf = 0.17) were separated completely from phosphatidylcholine (Rf = 0.39) when the distance of the solvent front from the origin was 17 cm. After drying, the thin-layer chromatography plates were exposed to iodine vapor for 1 hr. The spots corresponding to the lipid standards were marked and scraped off the plates and the radioactivity was determined by liquid scintillation counting. About 95% of the radioactivity was recovered. Phospholipase A_2 activity was determined by the release of [^{14}C] lysophosphatidylcholine from [^{14}C]phosphatidylcholine. PC-PLC activity was determined by the release of [^{14}C]DAG from [^{14}C]phosphatidylcholine. Depending upon the substrate used and the activity being assayed, the product spots usually gave count rates of 2,000–20,000 cpm.

Analysis of Effects of DHA-VC and DHA-cho on PC-PLC and Phospholipase A_2 in Crude Cell Extracts of *Bacillus cereus*. At the time of the experiments, stocked 22.4 μCi of [^{14}C]phosphatidylcholine were suspended in 100 μl of dH_2O and then sonicated in a bath sonicator for 2 min at room temperature. Fifty micrograms of membrane fraction proteins or phospholipase C- or phospholipase A_2-rich proteins from *B. cereus* were incubated with 50 μl of DHA-VC and DHA-cho (120 μg/ml) (giving a final dose of 20 μg/ml), 100 μl of reaction buffer, 100 μl of [^{14}C]phosphatidylcholine solution, and 50 μl of 12 mM $CaCl_2$ for 2 hr at 37°C. The content of the reaction buffer was described in Analysis of Phosphatidylcholine Hydrolysis, above. After incubation, the same procedure as that described in Analysis of Phosphatidylcholine Hydrolysis was performed.

Analysis of Phospholipid Labeled with [^{14}C]Choline or [^{3}H]Myristate. Cells (2×10^5) were labeled with 2 μCi of [^{14}C]choline (sp act 50–60 mCi/mmol) or 10 μCi of [^{3}H]myristrate (sp act 87.5 mCi/mmol) for 48 hr. The last 24 hr of labeling was performed in serum-free medium. The labeled cells were washed once with PBS and then exposed to various doses of DHA-VC for 2 hr. Reactions were terminated by removing the supernatants, and the cells were washed three times with cold medium containing unlabeled 1 mM choline. Then, 2 ml of ice-cold methanol were added and the cells were transferred to glass tubes after a 10-min incubation at 4°C. The washed culture dish was rinsed twice with 1 ml of ice-cold methanol, and this solution was added to the glass tubes mentioned above. Two milliliters of chloroform were then added and the extracts were left for 1 hr at 4°C, followed by centrifugation at 400g for 10 min. Organic phases were dried under N_2 gas and lipids were fractionated by thin-layer chromatography using the following solvent systems. For fractionation of different phospholipids, chloroform/methanol/concentrated ammonia (65:25:4) (v/v/v) was used for the first-dimension and chloroform/acetone/methanol/acetic acid/water (30:40:10:10:5) (v/v/v/v/v) for the second-dimension. The spot corresponding to each lipid located by autoradiography was scraped off the plate and the radioactivity was measured by a liquid scintillation counting.

[^{3}H]Phorbol Dibutyrate Binding to PC-14 Cells. Cells (1×10^6 cells/ml) were incubated for 30 min at 37°C in medium containing 20 nM [^{3}H]phorbol dibutyrate ([^{3}H]PBt_2) (sp act 17.6 Ci/nmol). Cell suspensions were exposed to various concentrations of docosahexaenoic acid analogs for 2 hr. After exposure, all samples were filtered onto 0.65-μm (pore size) Durapore membranes (Nihon Millipore Kogyo, Yonezawa, Japan). The membranes were washed three times with PBS and the radioactivities were measured in a Beckmann (Tokyo) LS3801 liquid scintillation counter. The results are expressed in terms of specific PBt_2 binding, which is the difference between bindings in the absence and the presence of 10 μM unlabeled PBt_2. All experiments were performed in duplicate and the data are presented as the means of at least three independent experiments.

Time Course of Effects of DHA-VC and DHA-cho on Protein Kinase C Activity. PC-14 cells were exposed to 20 μg/ml of DHA-VC or DHA-cho for the indicated times, and sampled for protein kinase C assay. The protein kinase C activity was measured by the nonradioisotopic protein kinase assay (NRPK assay system; Medical and Biological Laboratory Co. Ltd., Nagoya, Japan).

DNA Synthesis. Cells (2×10^6) were treated with 20 μg/ml of DHA-VC or DHA-cho or PBS as the control for 2 hr. After incubation the drugs were removed and then the cells were incubated in a humidified atmosphere of 5% CO_2 and 95% air in the complete medium for 0 to 18 hr. At each time point, cells were resuspended in 1 ml of fresh complete medium containing 2 μCi/ml of [^{3}H]thymidine (sp act 6.7 Ci/mmol) and incubated for 30 min to produce radiolabeled DNA. The cells were then collected to a 15-ml centrifuge tube and were rinsed twice with ice-cold PBS. We added 10 μl of horse serum as a carrier and 5 ml of

10% ice-cold trichloroacetate and mixed well. This mixed solution was incubated on ice for 15 min, and the precipitate was collected by centrifugation at 1500*g* for 10 min at 4°C. Two hundred microliters of folic acid (Wako Pure Chemical Co., Osaka, Japan) were added to solubilize the pellets. The radioactivity was measured in a liquid scintillation counter.

Results

Effect of DHA-VC and DHA-cho on Growth Inhibition of Human Lung Cancer Cell Lines. Growth inhibition by DHA-VC and DHA-cho was examined by 3-[4,5-dimethylthiazol 2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay and IC_{50} values for DHA-VC and DHA-cho are shown in Table I. DHA-VC and DHA-cho had similar growth-inhibitory effects on the small cell lung cancer cell line (H69) and the non-small cell lung cancer cell lines PC-7, PC-9, and PC-14.

Changes in PC-PLC Activity in DHA-VC-Treated Cells. Formation of phosphatidic acid and DAG by stimulated cells can occur through several distinct pathways. Phosphatidylcholine can be hydrolyzed by PC-PLC to yield DAG as described above. The resulting DAG is then phosphorylated by DAG kinase to phosphatidic acid (10). Phosphatidic acid is also formed by direct action of phospholipase D on phosphatidylcholine (11–15). Sphingomyelin is formed by phospholipase D. Phosphatidic acid is thus formed from phosphatidylcholine and DAG by phospholipase D and DAG kinase. On the other hand, phospholipase A_2 forms lysophosphatidylcholine and arachidonic acid from phosphatidylcholine (16). We examined whether DAG production was affected by DHA-VC or DHA-cho treatment. Production of phosphatidic acid and sphingomyelin in cells treated with DHA-VC or DHA-cho was lower than that of DAG and lysophosphatidylcholine (data not shown). We compared the productions of DAG (and phosphatidic acid) and lysophosphatidylcholine before and after treatment with various doses of DHA-VC or DHA-cho. The DAG production in cells after treatment with DHA-VC or DHA-cho showed a dose-dependent increase in comparison with untreated cells (Fig. 2A). On the other hand, lysophosphatidylcholine production in cells treated with DHA-VC or DHA-cho was dose-dependently inhibited in comparison with untreated cells (Fig. 2B). After DHA-VC or DHA-cho treatment, there was no apparent change in sphingomyelin production and a slight increase in phosphatidic acid production (data not shown). Considering the fact that phosphatidic acid is both a phospholipase-D-mediated hydrolysis product of phosphatidylcholine and also a DAG kinase-mediated phosphorylation product of DAG, it is unlikely that phospholipase D activity would have been affected by DHA-VC or DHA-cho treatment. In addition, the fact that the ratio of [^{14}C]phosphatidic acid to [^{14}C] DAG remained constant before and after DHA-VC or DHA-cho treatment suggested that DAG kinase also was not affected by DHA-VC or DHA-cho treatment (data not shown). Therefore, we speculate that production of DAG (plus phosphatidic acid) and lysophosphatidylcholine from phosphatidylcholine reflect PC-PLC and phospholipase A_2 activity, respectively.

Table I. IC_{50}s of DHA-VC and DHA-cho with Continuous Exposure

Cell line	IC_{50}[a] (μg/ml)	
	DHA-cho[b]	DHA-VC[c]
PC-9	103.4 ± 22.5*	53.8 ± 23.5
PC-14	97.0 ± 18.3†	68.0 ± 18.5
PC-7	45.3 ± 10.2‡	55.3 ± 18.8
H69	42.9 ± 11.5‡	54.3 ± 14.5

[a] Drug concentration that inhibits cell growth by 50%.
[b] Each value is the mean of four independent experiments. Significance in the difference of the IC_{50} value by unpaired Student's *t* test: * versus † and † versus ‡. $P < 0.1$.
[c] There was no significant difference in the IC_{50} values of DHA-VC between all cell lines.

Effect of DHA-VC and DHA-cho on PC-PLC in Membrane Cell Extract. To confirm that DHA-VC and DHA-cho affects the DAG production mediated by PC-PLC, we measured each enzyme activity in membrane fractions from PC-14 cells (Fig. 3). A dose-dependent increase of PC-PLC activity was observed in membrane fractions in the presence of DHA-cho or DHA-VC. In the presence of 20 μ*M* DHA-VC, PC-PLC activity was increased in PC-14 cells by 6.5-fold. Addition of 20 μ*M* DHA-cho induced a 5.1-fold increase of PC-PLC activity in PC-14 cells. These results suggest that DHA-VC and DHA-cho activate PC-PLC, and the increased activity of PC-PLC increases the amount of DAG. We also examined these enzyme activities in homogenates of whole cells. Dose-dependent activation of PC-PLC activity by these DHA analogs was also observed (data not shown). These findings are consistent with data demonstrating increased DAG production in cells treated with DHA analogs (Fig. 2A).

Effect of DHA-VC on Semipurified PC-PLC and Phospholipase A_2. To explore the possible mechanism of DHA-analog-induced PC-PLC activation, we examined the effect of DHA analogs on the activity of phospholipase C semipurified from *Clostridium welchii*. The results shown in Figure 4A indicate that DHA-VC increased the production of DAG mediated by PC-PLC in a dose-dependent manner. DHA-VC (at 100) μg/ml increased PC-PLC activity significantly. On the other hand, DHA-VC inhibited phospholipase A_2 activity (ED_{50} = 800 μg/ml) (Fig. 4B).

Effect of DHA-VC on Phosphatidylcholine Turnover. To examine the effect of DHA-VC on PC-PLC-mediated hydrolysis of PC, PC-14 cells were labeled with [^{14}C]choline or [^{3}H]myristate for 48 hr and then

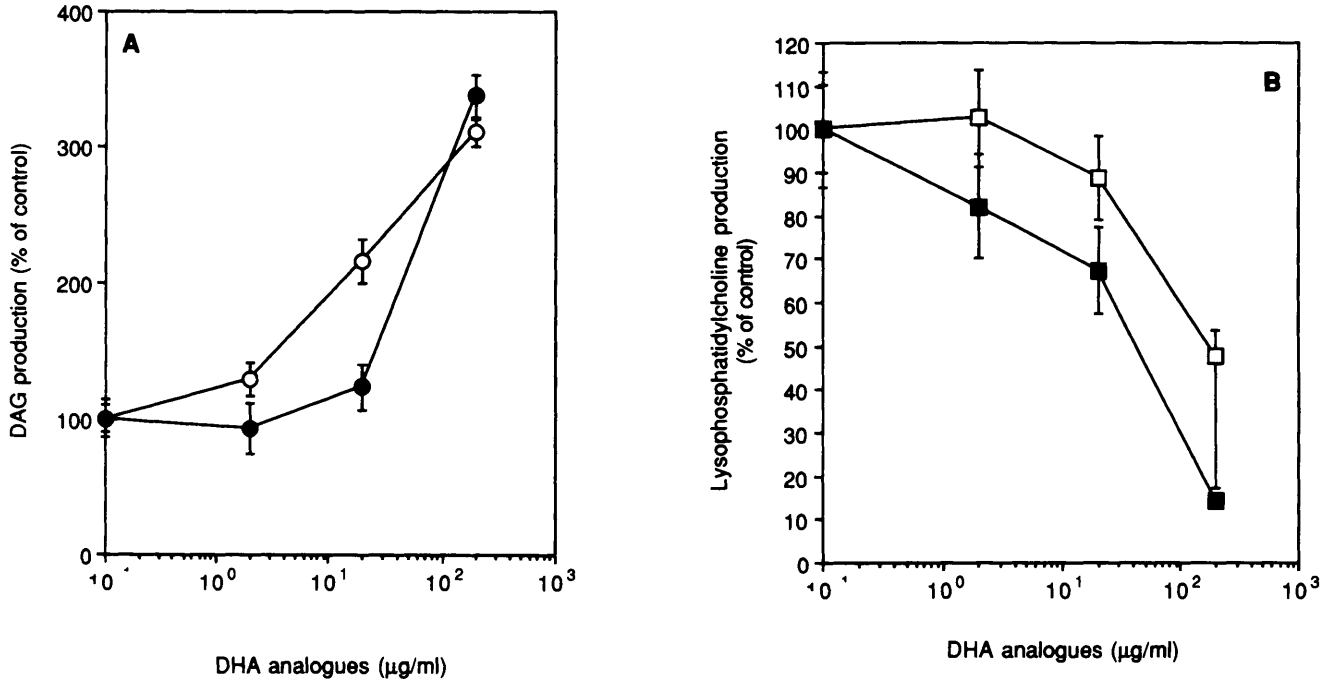


Figure 2. DAG and lysophospatidylcholine production from PC in DHA-VC- and DHA-cho-treated PC-14 cells. Fifty micrograms of membrane fraction proteins extracted from PC-9 cells using several concentrations of either DHA-VC or DHA-cho were incubated for 2 hr with 22.4 μCi of [^{14}C]PC in an appropriate reaction buffer. ^{14}C-Labeled lipids were extracted and analyzed by thin-layer chromatography. (A) DAG mass was obtained from the standard curve, and the results (triplicate determinations in two experiments) are expressed as DAG in DHA-VC (●)- and DHA-cho (○)-treated cells as a percentage of DAG in control PC-14 cells, which contained 1.5 nmol (900 cpm) of DAG per 2×10^6 cells. (B) Lysophosphatidylcholine mass was obtained from the standard curve, and the results (triplicate determinations in two experiments) are expressed as lysophosphatidylcholine in DHA-VC (■)- and DHA-cho (□)- treated cells as a percentage of DAG in control PC-14 cells, which contained 8.7 nmol (5200 cpm) of lysophosphatidylcholine per 2×10^6 cells.

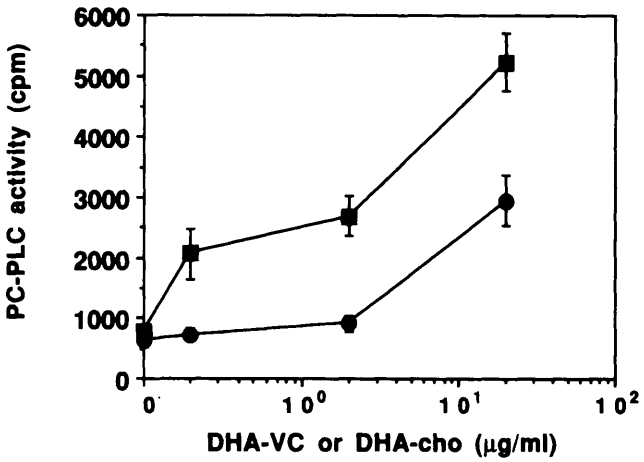


Figure 3. Dose response of the effect of DHA-VC and DHA-cho on PC-PLC in the membrane fraction of PC-14 cells. Membrane fraction was extracted from intact PC-14 cells. Fifty micrograms of membrane fraction proteins were incubated for 1 hr with various doses of DHA-VC (●) or DHA-cho (■) and 22.4 μCi of [^{14}C]phosphatidylcholine in an appropriate reaction buffer. PC-PLC activities were measured using [^{14}C]PC. The basal phosphatidylcholine level was 18,000–20,000 cpm. PC-PLC activity was expressed as the radioactivity of [^{14}C]DAG.

treated with various doses of DHA-VC for 2 hr. The last 24 hr of labeling was performed in serum-free medium. After 48 hr of labeling, the levels of ^{14}C-labeled and ^{3}H-labeled phosphatidylcholine became saturated (data not shown). The results shown in Figure 5, A and B indicated that the levels of ^{14}C- and ^{3}H-labeled phosphatidylcholine were constant after 2 hr of DHA-VC treatment, which suggests that DHA-VC treatment did not affect phosphatidylcholine turnover. Using [^{3}H]myristate, we were also able to monitor ^{3}H-labeled DAG (Fig. 5C). The incorporation of [^{3}H]myristate into DAG was increased in a dose-dependent manner by exposure to DHA-VC, which suggests that DHA-VC treatment caused an increase in DAG production. This result was consistent with the finding that DHA-VC increased DAG production, as shown in Figure 4A.

Effect of DHA-VC on Protein Kinase C Activity. We examined whether an increase of DAG affects protein kinase C activity. We observed an increase of [^{3}H]PBt$_2$ binding to the cells in the late phase after DHA-VC or DHA-cho treatment (Fig. 6A). Two hours after the start of the treatment, [^{3}H]PBt$_2$ binding was significantly ($P < 0.01$) increased in comparison with the control.

Protein kinase C activity determined by NRPK

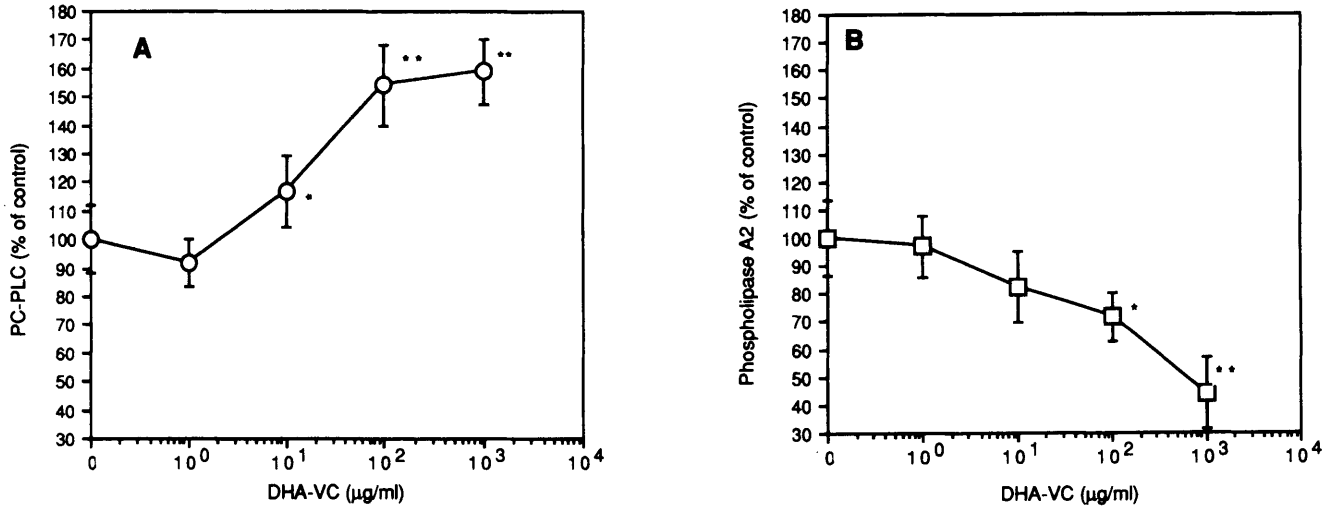


Figure 4. Effect of DHA-VC on semipurified PC-PLC and phospholipase A_2 from *C. welchii*. (A) Ten units of phospholipase C and (B) 5 units of phospholipase A_2 activity with several doses of DHA-VC were measured as described in Materials and Methods. Asterisks indicate a significant difference between DHA-VC exposed and control by Student's *t* test (**$P < 0.01$, *$P < 0.05$, respectively).

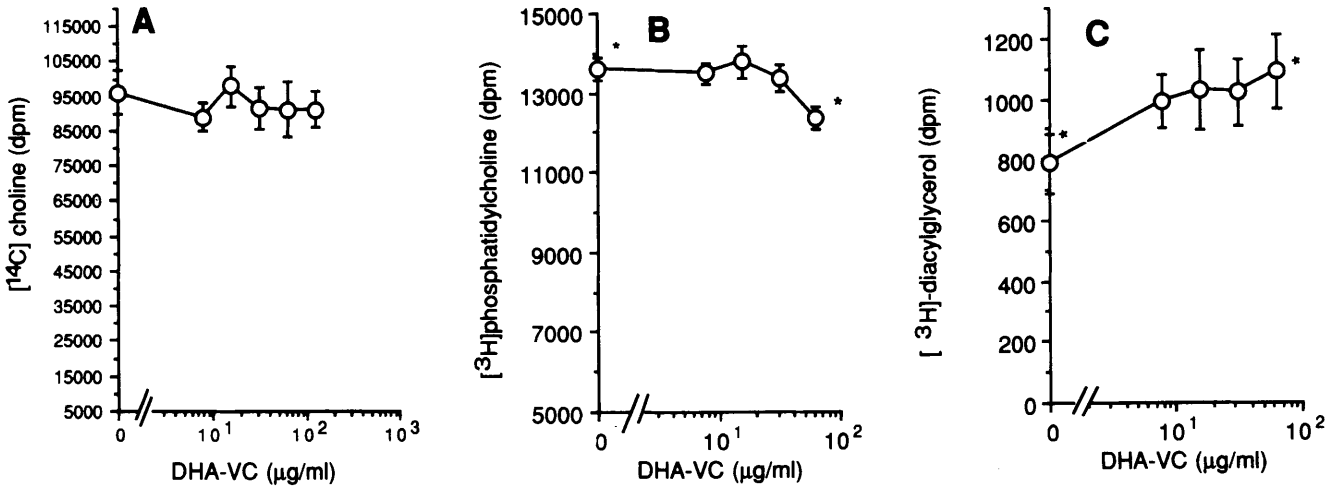


Figure 5. Dose response of the effect of DHA-VC on phosphatidylcholine labeled with (A) [^{14}C] choline and (B, C) [^{3}H]myristate (A) PC-14 cells (2×10^5 cells) were preincubated for 48 hr with 2 μCi of [^{14}C]choline, then exposed to various doses of DHA-VC for 2 hr. For the control experiments, PBS was added to the cells. After DHA-VC treatment, labeled lipids were extracted and analyzed by thin-layer chromatography. Basal [^{14}C]choline incorporation into phosphatidylcholine was 104,000 cpm per 2×10^5 cells. (B, C) PC-14 cells (2×10^5 cells) were preincubated for 48 hr with 2 μCi of [^{14}C]myristate, then exposed to various doses of DHA-VC for 2 hr. After DHA-VC treatment, the labeled lipids were extracted and then analyzed by thin-layer chromatography. Basal [^{14}C]choline incorporation into phosphatidylcholine and DAG was 13,500 cpm and 800 cpm per 2×10^5 cells, respectively. The results were expressed as radioactivities of [^{3}H]phosphatidylcholine and [^{3}H]DAG. The bars indicate SD values. Asterisk indicates a significant difference by unpaired Student's *t* test ($P < 0.01$).

assay was also increased by the addition of DHA-VC or DHA-cho (Fig. 6B).

Inhibition of DNA Synthesis after DHA-VC or DHA-cho Treatment. We determined the sequence of events (PC-PLC activation and inhibition of DNA synthesis) occurring in the cells after DHA-VC or DHA-cho treatment (Fig. 7). The significant inhibition of DNA synthesis was observed 12 hr after the beginning of incubation. It appeared that DHA-VC- or DHA-cho-induced phosphatidylcholine hydrolysis took place before the significant inhibition of DNA synthesis occurred.

Discussion

Protein kinase C has been demonstrated to be an important mediator of transmembrane signaling. *In vivo*, it is activated by increased DAG levels generated from the stimulated hydrolysis of phosphoinositides. Phosphatidylinositol 4,5-bisphosphate hydrolysis has been observed in response to a variety of agonists, including growth factors, hormones, and neurotransmitters (17). However, recent studies have demonstrated the existence of another phospholipid pathway leading to DAG production (18, 19). PC-PLC-mediated hydrolysis of phosphatidylcholine is now thought to be

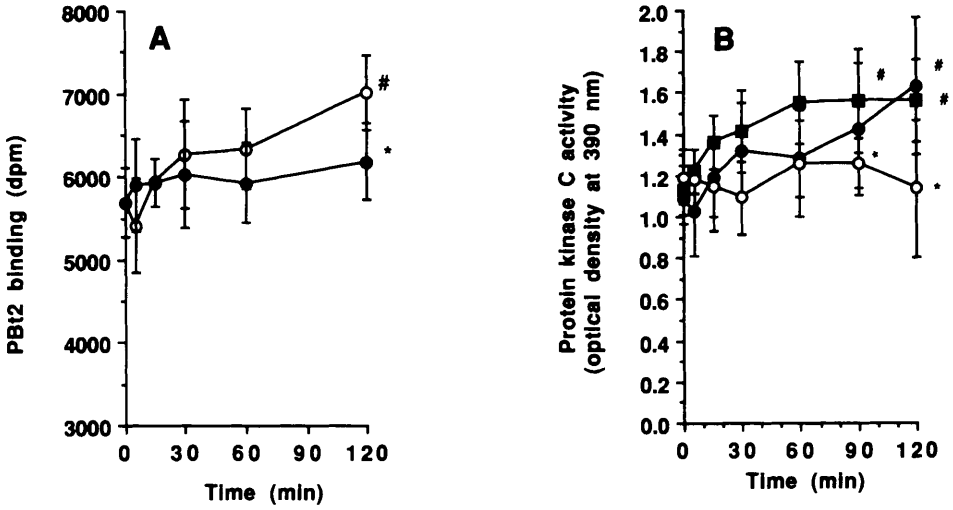


Figure 6. A The effect of DHA-VC on PBt_2 binding to PC-14 cells. PC-14 cells were preincubated for 30 min with 20 n*M* [^{3}H]PBt_2. Cells were exposed to 20 μg/ml of DHA-VC (○) or PBS (●) for the indicated times and then were taken for [^{3}H]PBt_2 binding assay. B Time course of the effect of DHA-VC on PKC activity. PC-14 cells were exposed to 20 μg/ml of DHA-VC (■), DHA-cho (●), or PBS (○) for the indicated times, and sampled for protein kinase C assay (non-radioisotopic protein kinase [NRPK] assay system). Each value is the mean of four independent experiments. The bars indicate SD. The significance in the difference of the protein kinase C activity between DHA-VC- or DHA-cho-treated and control (PBS treated) was tested by unpaired Student's *t* test: * versus #; $P < 0.01$.

another important source of DAG (20, 21). We have demonstrated that the treatment of water-soluble docosahexaenoic acid analog caused an increase in PC-PLC activity thus resulting in the increase of DAG, but did not affect phosphatidylcholine turnover in a human non-small cell lung cancer cell line. Phosphatidylcholine-derived DAG has been shown to differ from phosphatidylinositol 4,5-bisphosphate-derived DAG in its fatty acid composition (22) and functions (23, 24). In general, DAG generated from phosphatidylcholine are considered to accumulate more slowly than DAG generated by the hydrolysis of phosphatidylinositol 4,5-bisphosphate (25). It will be important to elucidate the effect of DAG derived from phosphatidylcholine on the protein kinase C and the following physiologic event.

DAG is considered to be an important intermediate in signal transduction pathways, regulating cell growth and transformation (26), and most studies have focused on its role in the regulation of cell proliferation. Issandou *et al.* (27) reported that diacylglycerol 1,2-diotanoyl-*sn*-glycerol showed a growth-inhibitory effect on the MCF-7 breast cancer cell line, and that this effect mimicked the action of phorbol ester. We have demonstrated previously that cisplatin, which is one of the potent anticancer agents, increased PC-PLC and DAG productions (28). These results suggest that DAG has a possible role in the negative regulation of cell proliferation. The activation of PC-PLC by DHA-VC described in this paper might also affect cancer cell growth.

DHA-VC-induced PC-PLC activation was observed even when we used semipurified phospholipase C from *C. welchii*. Although little information is available regarding the difference in structure and function of PC-PLC between mammalian and bacterial cells, DHA-VC is considered to have wide PC-PLC-activation activity. It would be very useful to examine the difference in the effect of DHA-VC on PC-PLC species between normal and tumor cells.

On the other hand, DHA-VC inhibited the activity of phospholipase A_2 (Figs. 2B, 4B), which suggests that inhibition of phospholipase A_2 might have some role in docosahexaenoic acid-induced growth inhibition.

Almost all of the previous studies investigating the relationship between the cytotoxicity of anticancer agents and signal transduction pathways have focused only on changes in protein kinase C and/or phosphatidylinositol turnover. However, data from the present study suggest that phosphatidylcholine metabolism might have an important role in drug-induced cytotoxicity.

DNA is not the accepted target for DHA-VC-induced cytotoxicity, and recent evidence has shed doubt on the view that DNA synthesis is the critical process affected by several anticancer agents (29). We determined the sequence of events including PC-PLC activation and inhibition of DNA synthesis occurring in cells after DHA-VC treatment. Considering the finding that 2 hr of DHA-VC treatment caused PC-PLC activation and that significant inhibition of DNA synthesis occurred 12 hr after 2 hr of treatment with DHA-VC (20 μg/ml) (Fig. 6), it appeared that DHA-VC-induced PC-PLC activation or phosphatidylcholine hydrolysis took place before any significant inhibition of DNA synthesis. Therefore, the effect of DHA analogs on signal transduction pathways might play an important role in DHA-VC-induced cytotoxicity.

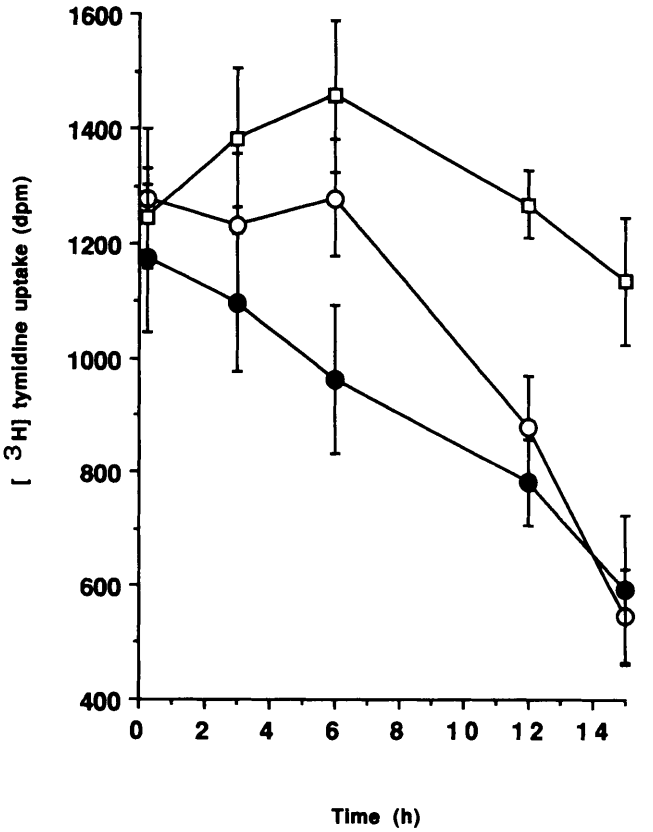


Figure 7. Inhibition of DNA synthesis in PC-14 cells at various times after 2 hr of treatment with DHA-VC or DHA-cho. PC-14 cells (2×10^6 cells) were treated for 2 hr with 20 μg/ml of DHA-VC (○), DHA-cho (●), or PBS (□). After 2 hr, drugs were removed and then cells were incubated in the complete medium for 0 to 18 hr. Cells were labeled with 2 μCi of [^{3}H]thymidine for 30 min at indicated time points. DNA synthesis was measured by determination of [^{3}H]thymidine incorporation.

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