Docosapentaenoic acid (22:5, n-3), an elongation metabolite of eicosapentaenoic acid (20:5, n-3), is a potent stimulator of endothelial cell migration on pretreatment in vitro

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Summary Endothelial cell (EC) migration plays an important role in wound repair of blood vessels. We have previously reported that eicosapentaenoic acid (EPA; 20:5, n-3) pretreatment stimulates migration of ECs but not smooth muscle cells. In the present study, we used the modified Boyden chamber technique to investigate whether the stimulative effect of EPA pretreatment on EC migration is caused by EPA itself or by some metabolites of EPA. When ECs were treated with EPA (5 μg/ml) for 2 days, EPA was predominantly elongated to docosapentaenoic acid (DPA; 22:5, n-3), with little docosahexaenoic acid (DHA; 22:6, n-3) being formed. Direct pretreatment of ECs with DPA (0.01–1.0 μg/ml) resulted in a dose-dependent increase in migration in response to fetal bovine serum. Moreover, maximum stimulation of EC migration by DPA pretreatment (0.5 μg/ml) was achieved at a concentration one-tenth of that required for maximal stimulation by EPA pretreatment (5.0 μg/ml), indicating that DPA is a potent stimulator of EC migration. We have demonstrated by lipid analysis that direct DPA pretreatment (0.5 μg/ml) sufficiently increased the absolute quantity of phospholipids of ECs. Cyclooxygenase inhibitor and lipoxygenase inhibitor did not abolish the stimulative effect of DPA pretreatment on EC migration. In contrast to EC migration, DPA pretreatment had no effect on smooth muscle cell migration. Together these data suggest that the stimulative effect of EPA on EC migration occurs via DPA, and that DPA may act as a powerful anti-atherogenic factor.

INTRODUCTION

Many factors such as hypercholesterolaemia,[1] hypertension[2] and oxidatively modified low-density lipoprotein[3] induce endothelial cell (EC) injury, which results in various pathological disorders including atherosclerosis and thrombosis. EC migration and proliferation are important processes in the control of wound-healing responses of blood vessels. EC migration and proliferation also play a central role in the healing following traumatic injury due to balloon catherization, graft placement or organ transplantation.

Eicosapentaenoic acid (EPA; 20:5), an n-3 polyunsaturated fatty acid (PUFA) present in fish oils, has been shown to have a protective effect against coronary heart disease, thrombosis and inflammatory processes.[4-7] In plasma, EPA reduces the triacylglycerol concentration and the production of low-density lipoproteins.[6,8] EPA also reduces platelet aggregation by decreasing the conversion of arachidonic acid (AA; 20:4, n-6) to thromboxane A_2 (TXA_2) and by itself being converted to prostaglandin D_3 (PGD_3) an anti-aggregatory substance.[9,10]

While there is general agreement regarding the effects of EPA on platelets, the available information regarding vascular tissue is less certain. For example, some investigators have reported that EPA did not inhibit the conversion of AA to PGI_2 by umbilical blood vessels,[11] and that EPA actually appeared to increase PGI_2 production in

rat aorta.[12] Conversely, others have observed that EPA reduces PGI_2 production and that EPA itself is not converted to PGI_3 in rat smooth muscle cells (SMCs),[13] human ECs[14] and bovine ECs.[15]

We previously reported[16] that EPA specifically enhanced EC migration and showed that the preventive effect of EPA on atherosclerosis may be partly due to its stimulative effect on EC migration. We discovered by lipid analysis that a large amount of docosapentaenoic acid (DPA; 22:5, n-3), the elongated product of EPA, existed in EC phospholipids.[16] Other studies have also reported an increase in the DPA content of phospholipid of bovine[15] and human umbilical vein[14] ECs following EPA pretreatment. In general, as EPA and docosahexaenoic acid (DHA; 22:6, n-3) are major components of fish oil, both substances are often used to investigate the effects of fish oil. However, there are no reported studies examining the physiological role of DPA alone. In this paper, we have demonstrated that the stimulative effect of EPA on EC migration may be due to DPA. Moreover, the potency of DPA stimulation of EC migration is ten-fold greater than that of EPA.

MATERIALS AND METHODS

Chemicals

EPA and DHA (purity > 99%) were purchased from Sigma Chemical Co. (St Louis, MO, USA). DPA (purity > 97%) was obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA). In order to prevent oxidation, the fatty acids were dissolved in ethanol, glassed and capped under nitrogen and stored at −80°C after each experiment. Eagle's minimum essential medium (MEM) was purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from Sanko Junyaku Co. Ltd (Tokyo, Japan). Caffeic acid (a lipoxygenase inhibitor), indomethacin (a cyclooxygenase inhibitor) and N-ethylmaleimide (an elongation enzyme inhibitor) were from Sigma Chemical Co.

Cell culture and treatment with PUFAs

ECs were isolated from bovine carotid arteries and maintained as described previously.[17] ECs at passage numbers 7–14 were used for the present study.

Bovine aortic SMCs were isolated from medial explants of thoracic aortae by the methods of Ross[18] and maintained as described previously.[16] SMCs were used for experiments at passage numbers < 15.

The method for treatment with PUFAs has been reported previously.[16] Briefly, for migration assays ECs or SMCs at confluence in 28-cm² plastic dishes (Falcon, Oxnard, CA, USA) were treated with PUFAs in 4 ml of 10%

FBS-MEM. The medium was renewed after 24 h. Ethanol stock solutions of PUFAs were diluted to various concentrations with ethanol and added directly to culture medium supplemented with 10% FBS. For control cultures, the same concentration of vehicle (0.1% ethanol) was added. After treatment of the cells with PUFAs for 48 h, the incubations were terminated by removing the medium, and the cell layers were washed with MEM. The trypsinized cells were used for migration assays.

For lipid analysis, ECs cultured in 75-cm² plastic dishes were treated with PUFAs in 9 ml of 10% FBS-MEM. The incubation time and the termination procedure were the same as for cells prepared for the migration assays.

Migration assays

The migration assay methods have been reported previously.[16] Briefly, the modified Boyden chamber technique was performed using a 48-well microchemotaxis chamber (Neuroprobe, MD, USA). The thickness and pore size of nucleopore filters were 10 μm and 8 μm, respectively. In order to use a common stimulator of EC and SMC migration, the lower chambers of wells were filled with 26 μl of 10% FBS-MEM. Cells (2 × 10⁴/well), which had been pretreated with PUFAs for 2 days and suspended in 50 μl 2% FBS-MEM, were added to the upper wells.

The incubation time for migration was about 4 h. After fixation and staining, five high-power fields (HPF, ×400) in each of six replicate wells were examined to determine the number of cells that had migrated onto the lower surface of the filters. Cell migration was expressed as the total cell number in 5 HPF (total cells/5 HPF).

Each experiment was repeated 3 times. Although there was day-to-day variation in the absolute number of migrating cells and ECs lost their ability to migrate gradually with additional passages, similar results were obtained in each repeated experiment. The results presented in this paper are the representative results of one experiment performed on a single day.

Lipid analysis

After PUFA treatment, the fatty acid contents of cellular phospholipids were analyzed as reported previously.[16] Briefly, cell layers were scraped, harvested and the cellular lipids were extracted by the method of Bligh and Dyer.[19] The phospholipid fraction was isolated by thin-layer chromatography. The phospholipids were transesterified and separated by gas–liquid chromatography. The absolute quantity of fatty acids was calculated by adding methyl $C_{22:0}$ as an internal standard. The methyl $C_{22:0}$ peak did not overlap with any other peak in the gas–liquid chromatography profile.

Statistical analysis

The data were analyzed by Student's t-test.

RESULTS

Effects of EPA and DHA pretreatment on lipid content and migration activity of ECs

When ECs were treated with EPA (5 µg/ml) for two days, the DPA content of the EC phospholipids markedly increased, from 3.6 ± 0.2% to 15.9 ± 0.5%, in addition to the expected increase in EPA (Fig. 1A). Migration activity of EPA pretreated ECs was enhanced by FBS on microwell chemotaxis assay system and the stimulative ratio was twice the control ($P < 0.01$, Fig. 1B).

DHA treatment (5 µg/ml) for 2 days increased DHA content in EC phospholipids, from 5.6 ± 0.2% to 18.4 ± 0.3%, and also increased EPA content from 0.6 ± 0.1% to 4.3 ± 0.1% (Fig. 1A). However, DHA treatment did not change the DPA content of the ECs. In spite of the raised EPA content of the EC phospholipids, DHA pretreatment did not effect cell migration (Fig. 1B). Although we used various doses of DHA, no effect on EC migration was observed (data not shown). These results suggest that enhancement of EC migration may occur via DPA.

Effects of direct pretreatment with DPA on EC migration

Migration activity of ECs was examined after the cells were pretreated directly with DPA for 2 days. As shown in Fig. 2, DPA pretreatment stimulated EC migration dose-dependently, and the dose–response curve was bell-shaped. Significant stimulation of cell migration was observed for DPA concentrations of 0.05 µg/ml to 5.0 µg/ml and the maximum stimulation was at 0.5 µg/ml. The concentrations at over 10 µg/ml of any PUFA must be toxic in this system as morphological changes were observed in the ECs. We have previously reported that EPA induced its maximum stimulation of EC migration at the concentration of 5.0 µg/ml,[16] indicating that DPA pretreatment was ten-fold more potent than EPA pretreatment.

To compare the maximum effect on EC migration of DPA with that of EPA, we examined the effects of both PUFAs using cells from the same preparation. As shown in Fig. 3, 0.5 µg/ml of EPA pretreatment slightly enhanced EC migration. On the other hand, the stimulative effect by DPA pretreatment at 0.5 µg/ml was equivalent to that by EPA pretreatment at 5.0 µg/ml. Although day-to-day variation in the absolute number of migrating cells was observed, there was no significant difference between DPA (0.5 µg/ml) and EPA (5.0 µg/ml) pretreatments in any experiments.

We performed lipid analysis to measure the absolute quantities of DPA in the phospholipids of ECs pretreated
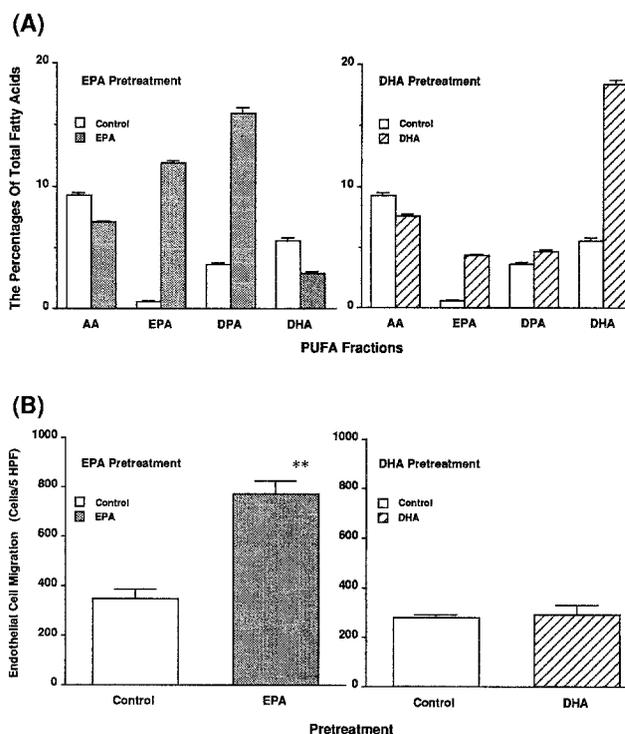

(A)

(B)

Fig. 1 Lipid analysis of EPA and DHA pretreated ECs and effects of these PUFAs on EC migration. ECs were cultured in MEM supplemented with 10% FBS. After inoculation, the ECs were exposed to medium containing 5 µg/ml of either EPA or DHA for 2 days as described in the text. (A) The fatty acid composition of the phospholipids was analyzed by capillary gas chromatography as described in Materials and Methods. (B) The migration activity of ECs pretreated with each PUFA was then assayed. Cells were suspended in MEM supplemented with 2% FBS in the upper chamber. The lower chamber was filled with culture medium supplemented with 10% FBS. The migration of the cells was determined as described in the text. Columns and bars represent mean ± SEM of the results of six replicate wells (**$P < 0.01$).

with either EPA at 5.0 µg/ml, or with DPA at 0.5 µg/ml. After treatment with EPA or DPA for 2 days, cell layers were harvested and the phospholipids extracted. Separation of phospholipids was carried out by gas–liquid chromatography. Table 1 shows the absolute quantity of each PUFA in the phospholipids of the ECs. EPA pretreatment markedly increased the EPA content of EC phospholipids, by 15.2-fold compared to the control. The DPA content of EC phospholipids was also increased by EPA pretreatment, by 4.4-fold compared to the control. In spite of the much lower concentration used, DPA pretreatment at 0.5 µg/ml also increased the DPA content of EC phospholipids, by 2.4-fold compared to the control. The contents of EPA and DHA have slightly increased by DPA pretreatment as compared to the control.

In order to determine whether there is a direct effect of DPA on EC migration, DPA was added to the cell suspension exogenously just before migration assay. The upper chamber was filled with cell suspension in 2% FBS-MEM
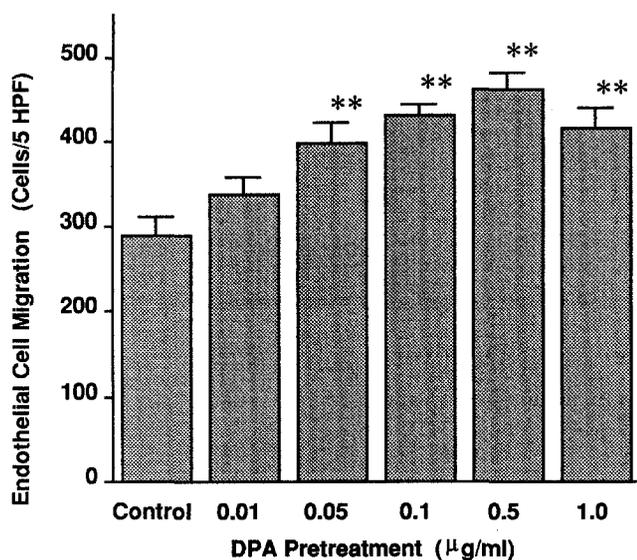
Fig. 2 Effect of DPA pretreatment at various concentrations on EC migration. ECs were inoculated onto culture dishes and cultured in medium supplemented with 10% FBS. After inoculation, the medium was replaced with culture medium containing 10% FBS and various concentrations of DPA. The medium was replaced with fresh DPA enriched medium after 24 h. The migration activity of the DPA-enriched ECs was then assayed. In the assay, the upper chamber was filled with the cell suspension in culture medium supplemented with 2% FBS, and the lower chamber was filled with culture medium supplemented with 10% FBS. The migration of the cells was determined as described in the text. Columns and bars represent means ± SEM of the results of six replicate wells (**$P < 0.01$).
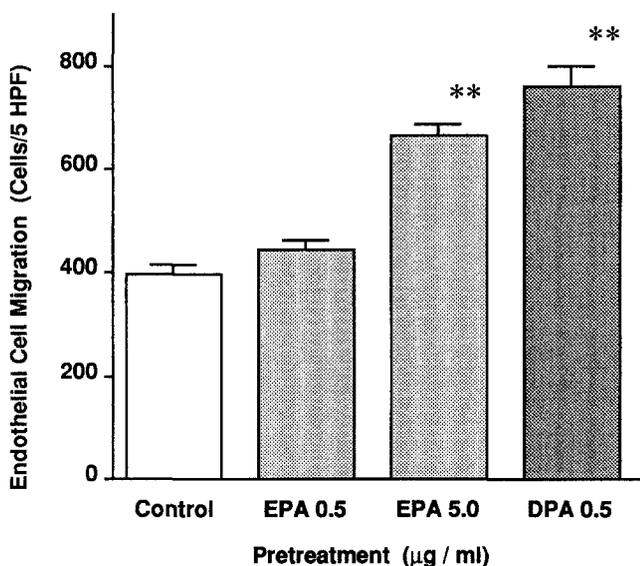

Fig. 3 Comparative effects of maximum stimulation of EPA and DPA on EC migration. After EC inoculation, the cells were exposed to medium containing 0.5 μg/ml EPA and 5.0 μg/ml EPA or 0.5 μg/ml DPA for 2 days as described in the text. The migration activity was then assayed. Cells were suspended in culture medium supplemented with 2% FBS in the upper chamber. The lower chamber was filled with culture medium supplemented with 10% FBS. The migration of the cells was determined as described in the text. Columns and bars represent means ± SEM of the results of six replicate wells (**$P < 0.01$).

Table 1 The absolute quantities of PUFA in phospholipids of ECS pretreated with EPA and DPA

Fractions	Control	Pretreatment EPA (5 μg/ml)	DPA (0.5 μg/ml)
EPA	3.46 ± 0.48	52.73 ± 10.4	7.25 ± 1.06
DPA	8.66 ± 1.40	38.22 ± 7.03	21.13 ± 3.03
DHA	11.07 ± 1.55	18.64 ± 3.43	19.39 ± 2.63

Values are mean ± SD of the absolute quantity (ng/10^7 cells) of PUFA in the phospholipid fraction ($n = 5$). After ECs were pretreated with EPA and DPA for 48 h, the fatty acid composition of the phospholipids was analyzed by capillary gas chromatography. The absolute quantity was calculated by adding methyl $C_{22:0}$ as an internal standard as described in Materials and Methods.

containing various concentrations of DPA and the lower chamber was filled with 10% FBS-MEM. In this case DPA had no effect on EC migration, indicating that DPA incorporation into ECs is important for EC migration.

In the previous paper, we showed that EPA pretreatment made chemokinetic motility of ECs easier but that EPA was not a chemoattractant.[16] We also examined the chemoattractant effect of DPA on EC migration. The lower wells of the chamber were filled with MEM supplemented with 2% FBS containing various concentrations of DPA, and ECs without DPA pretreatment were added to the upper wells. As with EPA, DPA did not stimulate chemotactic movement of ECs (data not shown).

Effect of DPA on SMC migration

In our previous paper, EPA pretreatment did not affect SMC migration.[16] In the present study we investigated the cell-type specificity of DPA pretreatment by observing the effects of DPA pretreatment on SMC migration. SMCs were pretreated with various concentrations of DPA for 2 days. After the DPA pretreatment, the microwell chemotaxis assay was performed using FBS as a stimulator. As with EPA pretreatment, DPA pretreatment had no effect on SMC migration at any of the doses examined (the migrating SMCs of control, 0.1 μg/ml, 0.5 μg/ml, 1.0 μg/ml and 5.0 μg/ml DPA pretreatment were 54.2 ± 4.13, 47.5 ± 4.17, 52.2 ± 4.36, 55.0 ± 4.12, and 67.2 ± 4.22 cells/5HPF, respectively).

Effect of inhibitors on migration of ECs pretreated with DPA

EPA is converted to type 3 prostaglandins and thromboxanes by cyclooxygenase, and to hydroperoxyeico-sapentaenoic acid, hydroxyeicosapentaenoic acid and type 5 leukotrienes by lipoxygenases. Therefore we examined whether DPA metabolites generated by these enzymes were responsible for the stimulative effect of DPA on EC migration. Caffeic acid (10 μM, a lipoxygenase

inhibitor) and indomethacin (10 µM, cyclooxygenase inhibitor) were used in the migration assay, being added to both the upper and lower chambers. ECs pretreated with DPA at 0.5 µg/ml for 2 days were suspended in 2% FBS-MEM with or without inhibitor, and were added to the upper chamber. The lower chamber was filled with 10% FBS-MEM with or without inhibitor. As shown in Figure 4, the addition of caffeic acid or indomethacin had no effect on the migration of control ECs. Moreover, EC migration stimulated by DPA pretreatment showed no significant difference between inhibitor-free, caffeic acid-treated or indomethacin-treated groups. These results showed that neither cyclooxygenase nor lipoxygenase metabolites of DPA are involved in the stimulation of EC migration.

Although N-ethylmaleimide is an inhibitor of the enzyme which elongates EPA to DPA, it is not specific for this enzyme. We used N-ethylmaleimide for EC pretreatment in preliminary cell migration experiments. ECs were pretreated concomitantly with N-ethylmaleimide and EPA or DPA and migration activity was examined. DPA (0.5 µg/ml) and EPA (5.0 µg/ml) pretreatments increased EC migration to 188.9 ± 12.52 ($P<0.01$) and 214.4 ± 32.66 ($P<0.01$) percent of control values, respectively. N-ethylmaleimide alone did not affect migration of control cells (123.9 ± 7.52%). Concomitant pretreatment with EPA and N-ethylmaleimide abolished the stimulative effect of EPA (90.9 + 10.10%) on EC migration but

Fig. 4 The effect of caffeic acid and indomethacin on FBS-induced migration of ECs pretreated with DPA. ECs were pretreated with DPA (0.5 µg/ml) for 2 days as described in the text. The migration activity with or without inhibitors was then assayed. ECs were suspended in culture medium supplemented with 2% FBS in the upper chamber. The lower chamber was filled with culture medium supplemented with 10% FBS. Caffeic acid (10 µM, CA) or indomethacin (10 µM, INDO) was added to both upper and lower chambers. The migration of the cells was determined as described in the text. Open columns denote control ECs and hatched columns DPA-pretreated ECs. Columns and bars represent means ± SEM of the results of six replicate wells (*$P < 0.05$, **$P < 0.01$).

pretreatment with DPA and N-ethylmaleimide did not reduce the stimulative effect of DPA (193.8 + 16.12%, $P<0.01$). These results indicate that EPA must be elongated to have an effect in enhancing EC migration.

DISCUSSION

DPA is an elongated product of EPA. Many reports regarding the uptake of EPA have shown an increase in DPA contents of plasma and blood cells. For example, a diet with a mixture of EPA and DHA ethyl ester increased the DPA content of lipids in human plasma, platelets, monocytes and red blood cells over a period of 18 weeks.[20] Dietary injection of EPA ethyl ester also increased the content of DPA in rabbit low-density lipoprotein.[21] Administration of purified EPA to volunteers showed a significant rise in plasma and platelet fatty acid DPA content.[22]

A number of papers reporting in vitro experiments have revealed the possibility that vascular cells, bovine SMCs,[23] bovine ECs,[15] and human umbilical vein ECs[14] can elongate EPA to DPA. The work of Rosenthal and co-workers indicates that EPA was easily elongated, compared with AA, in human umbilical ECs[24] and fetal skin fibroblasts.[25] In human umbilical ECs elongation of incorporated ^{14}C-EPA is 70% by 24 h, whereas elongation of incorporated ^{14}C-AA is only 16% by 24 h.[24]

In contrast, the pathway from DPA to DHA by Δ^4 desaturation was found in a few cell types. Voss and co-workers[26] have studied the metabolism of DPA to DHA in rat liver and reported that the metabolism was independent of a Δ^4 desaturase and that this pathway involves the microsomal chain elongation of DPA to 24:5, n-3, followed by its desaturation to 24:6, n-3. This product is then metabolized to DHA via β-oxidation. Retinoblastoma cells are one of a few cell types which actively convert exogenous linolenic acid or EPA to DHA.[27,28]

In the present paper, we have demonstrated that EPA was easily converted to DPA (Fig. 1). As shown in Table 1, the increase in EPA content was about 50 ng/10^7 cells and the increase of DPA was about 30 ng/10^7 cells following EPA treatment (5 µg/ml for 2 days). In contrast, the increase in DHA content following EPA treatment was only 7 ng/10^7 cells. These results are supported by the data published by Garcia et al[24] who reported that umbilical vein ECs did not exhibit significant desaturation of DPA, and with the work of Spector et al[14] who showed that EPA treatment did not increase the DHA content of phospholipids in ECs. Our observation that a large amount of DPA was converted from EPA in ECs led us to hypothesize that the effect of EPA pretreatment on EC migration is not direct but occurs instead via DPA.

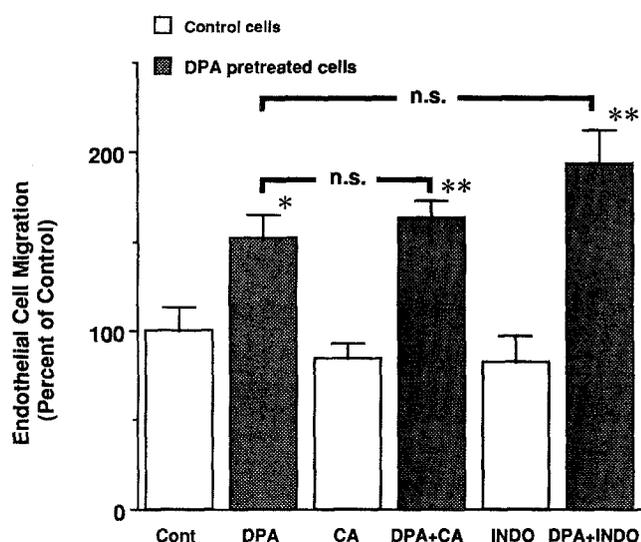As shown in Fig. 1B, DHA pretreatment was not able to stimulate the EC migration, whereas pretreatment with

EPA or DPA did stimulate the EC migration. However, the DPA concentration which produced maximal stimulation was one-tenth of the EPA concentration which gave maximal stimulation. It is interesting that the DPA content in phospholipids after the treatment with EPA at 5.0 μg/ml for 2 days was almost the same as after treatment with DPA at only 0.5 μg/ml (Fig. 3). These data strongly suggest that the stimulative effect of EPA pretreatment on EC migration is caused by DPA, and not by DPA metabolites produced by cyclooxygenases or lipoxygenases (Fig. 4).

In cases of atherosclerosis, EC migration is an important process in wound repair of blood vessels. In contrast, SMC migration plays a central role in the genesis of atherosclerosis. In the previous paper, we demonstrated that the migration of ECs was suppressed, but that of SMCs was stimulated by cholesterol, which is one of the risk factors of atherosclerosis.[23] Moreover, concomitant EPA and cholesterol treatment demonstrated that EPA abolished the effects of cholesterol on the migration of both cell types. The lipid analysis showed that the content of EPA and DPA in phospholipids of both cell types was increased by EPA treatment. These data indicate that the anti-atherogenic effects of EPA on both cell types may be expressed via DPA, although further studies are required to confirm this hypothesis. It is encouraging that Hodgson et al[29] reported that the DPA concentration in platelets was inversely associated with coronary artery disease in women.

The mechanism by which DPA enrichment stimulates EC migration is still unclear. Cell migration is dependent on cytoskeletal structures and adhesion molecules and lipids have a close relation with them. Bates et al[30] showed that PUFA induced in the expression of integrin receptors on neutrophils. Moreover, Ferrante et al[31] suggested that up-regulation of integrin receptors, induced by PUFA, may result in very tight attachment of neutrophils to the extracellular matrix, rendering them immobile. Although it is not known if these responses occur in other cell types, it is important to investigate the effect of n-3 and n-6 PUFAs on the expression of integrin receptors.

Recently, it has been reported that the signal transduction pathway for migration is different from that for proliferation in ECs and SMCs.[32,33] EC movement is mediated by a pertussis toxin-sensitive pathway regulating phospholipase A_2 activity, and only AA overcomes the pertussis toxin block.[32] We could not, however, show that AA pretreatment enhanced EC migration.[16] Further study of the relationship between cell migration and lipids, including DPA, is required to address this apparent discrepancy.

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