Turnover of eicosanoid precursor fatty acids among phospholipid classes and subclasses of cultured human umbilical vein endothelial cells

Hiroshi TAKAYAMA,* Michael H. KROLL,* Michael A. GIMBRONE, Jr.† and Andrew I. SCHAFER*‡

*Hemostasis Unit, Hematology Division, Department of Medicine, and †Vascular Research Division, Department of Pathology, Brigham and Women's Hospital, and the Departments of *Medicine and †Pathology, Harvard Medical School, Boston, MA 02115, U.S.A.

Using cultured human umbilical vein endothelial cells, in which phosphatidylcholine (PC) is equally pulse-labelled by various eicosanoid precursor fatty acids (EPFAs), we have studied the remodelling of EPFAs among the phospholipid classes and subclasses with and without activation, and the relationship of this remodelling process to the selective release of arachidonic acid (AA) by phospholipase A_2 -mediated cell stimulation. When endothelial cells are pulse-incubated with radiolabelled EPFA for 15 min, >80% of cell-associated radioactivity is present in phospholipids, among which >60% is found in 1,2-diacyl-*sn*-glycero-3-phosphocholine (diacyl PC). After removing unincorporated radioactivity, reincubation of the pulse-labelled cells for up to 6 h results in progressive decrease in EPFA-labelled diacyl PC, increase in AA- or eicosapentaenoic acid (EPA)-labelled 1-*O*-alk-1-enyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (plasmalogen PE) and increase only in AA-labelled 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (alkyl PC). This redistribution of radiolabelled phospholipids is not altered by the presence of excess non-radiolabelled EPFAs. When aspirin-treated EPFA-labelled endothelial cells are stimulated with ionophore A23187, a very selective release of AA is noted in comparison with eicosatrienoate (ETA) or EPA, accompanied by an equivalent decrease in AA-labelled diacyl PC and specific increase in AA-labelled plasmalogen PE and alkyl PC. These selective changes in AA radioactivity induced by A23187 are enhanced 2-fold by pretreating the AA-labelled cells with phorbol 12-myristate 13-acetate, which by itself induces no changes. The changes in radioactivity induced by A23187 without and with phorbol ester among the released AA, the diacyl PC and the plasmalogen PE are significantly correlated with each other. These results indicate that human endothelial cells incorporate EPFAs (AA, ETA, EPA) equally into diacyl PC but selectively release AA esterified into diacyl PC with specific remodelling into plasmalogen PE and alkyl PC.

INTRODUCTION

Arachidonic acid (AA) is metabolized into various biologically active eicosanoids via cyclo-oxygenase and lipoxygenase pathways [1]. In intact cells, a major determinant of eicosanoid synthesis is the availability of free AA, which is controlled by its esterification and de-esterification into and from complex lipids such as glycerophospholipids [2]. Although it is known that AA is non-uniformly distributed among specific cellular phospholipids [3,4] and is released selectively in response to physiological stimuli [2,5], the mechanisms for these processes are as yet incompletely understood.

Several schemes have been proposed to explain these processes. A specific arachidonoyl-CoA synthetase has been described in human platelets [6]. This enzyme could be responsible for the high-affinity esterification system of AA and other eicosanoid precursor fatty acids

(EPFAs) into 1-acyl-2-lysoglycerophospholipids in various cells [7–9]. A CoA-independent transacylase which catalyses the transfer of AA from 1,2-diacyl-*sn*-glycero-3-phosphocholine (diacyl PC) to ether-linked glycerophospholipids such as 1-*O*-alk-1-enyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (plasmalogen PE) and 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (alkyl PC) has been reported in human platelets [10,11] and rabbit macrophages [12,13]. This enzyme may play an important role in the redistribution of AA to the ether-linked glycerophospholipids which are known to be enriched in AA [14–16]. Ether-lipid-selective phospholipases which have been reported in sheep platelets [17] might cleave the *sn*-2 position of AA-enriched ether phospholipids and selectively release AA in response to physiological stimuli. The hydrolysis of phosphatidylcholine (PC) by phospholipase A_2 is responsible for the majority of released AA after physiological stimuli in most cell types

Abbreviations used: AA, arachidonic acid; EPFA, eicosanoid precursor fatty acid; diacyl PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; plasmalogen PE, 1-*O*-alk-1-enyl-2-acyl-*sn*-glycero-3-phosphoethanolamine; alkyl PC, 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine; PAF, platelet-activating factor; diacyl PE, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine; HEC, human endothelial cells; PBS, phosphate-buffered saline; EPA, eicosapentaenoic acid (ω -3); ETA, eicosatrienoic acid (ω -6); PMA, phorbol 12-myristate 13-acetate; alkyl PE, 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; NL, neutral lipid; FFA, free fatty acid; ETYA, 5,8,11,14-eicosatetraynoic acid.

‡ To whom correspondence and reprint requests should be addressed at: Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115, U.S.A.

studied [18–20]. However, the relationship between the selective release of AA by phospholipase A_2 and the specific remodelling of AA from PC into ether-linked phospholipids has not been previously examined in detail.

Endothelial cells have been known to produce important biologically active phospholipid-derived mediators, including prostacyclin, a potent vasodilator and inhibitor of platelet activation [21,22], and platelet-activating factor (PAF) [23,24]. We have recently described the pulse-labelling of cultured human endothelial cells (HEC) with EPFAs via a high affinity esterification system [9]. Phosphatidylcholine was found to incorporate a significantly greater amount of radiolabelled EPFAs than other phospholipids. Furthermore, in contrast to the preferential incorporation of AA into other phospholipid classes, PC exhibited no preference for EPFAs. This experimental model has now enabled us to study for the first time the specific remodelling of EPFA-labelled glycerophospholipids in both resting and stimulated cells, as well as the relationship of this remodelling process with the selective release of AA by phospholipase A_2 -like activity. We now report that a highly specific remodelling system for AA is operative in endothelial cells that have been labelled with EPFAs.

EXPERIMENTAL PROCEDURES

Materials

Calcium ionophore A23187 was obtained from Calbiochem; phorbol 12-myristate 13-acetate (PMA), phospholipase C (*Bacillus cereus*), benzoic anhydride, 4-dimethylaminopyridine and phospholipid standards including bovine brain phosphatidylethanolamine (containing diacyl- and plasmalogen-PE in an approximately 1:1 ratio) were from Sigma; alkyl PC was from Avanti Polar Lipids Inc. (Birmingham, AL, U.S.A.); Medium 199 was from Whittaker M.A. Bioproducts (Walkersville, MD, U.S.A.). All other materials were obtained from sources reported previously [9,22].

Human endothelial cell culture

Human endothelial cells (HEC) were isolated from umbilical veins and maintained in primary culture as previously described [25,26]; subcultured strains of HEC were propagated using endothelial cell growth factor (50–100 $\mu\text{g}/\text{ml}$; Collaborative Research Inc., Waltham, MA, U.S.A.) and porcine intestinal heparin (50–100 $\mu\text{g}/\text{ml}$; Sigma), and utilized at passages 2–4.

Labelling of cultured endothelial cells

Unless otherwise stated, all procedures were performed under sterile conditions. Monolayers of replicate plated cultures of HEC in 60 mm Petri dishes (total protein 0.2–0.3 mg) were washed three times with 2 ml of M199 including 1 mg of fatty-acid-free bovine albumin (Sigma)/ml, and then incubated with 200 nM- ^{14}C -labelled EPFAs (specific radioactivity 50–57 mCi/mmol) at 37 °C for 15 min. In some experiments, the monolayers of HEC were treated with aspirin (1 mM) for 30 min to inhibit cyclo-oxygenase before washing and labelling with [^{14}C]EPFAs. Analysis of radiolabelled fatty acids present both in the cell-free media and deacylated from HEC phospholipids demonstrated that >95% of all radiolabelled fatty acids remained unchanged after the

15 min incubation with HEC [9]. HEC monolayers were washed once, and 2 ml of the same medium or 2 ml of Dulbecco's phosphate-buffered saline (PBS) including 1 mg of fatty acid-free bovine albumin/ml were added.

Incubation of labelled endothelial cells with or without activation

The washed monolayers of radiolabelled HEC were incubated for up to 6 h at 37 °C in an atmosphere of air/ CO_2 (19:1). In the experiments with stimulants, monolayers of aspirin-pretreated and radiolabelled HEC in 2 ml of PBS plus albumin were used. Pretreatment of HEC with 50 μM -ETYA, an inhibitor of both cyclo-oxygenase and lipoxygenase, yielded the same results as the experiments reported using aspirin. The various agents were added directly to the monolayers. At each time point, the medium was removed and the monolayers were washed once with 2 ml of PBS plus albumin. Radioactivity in both the medium and washing solution was counted. Lipid extractions of HEC were performed as described previously [9].

Lipid analysis

Phospholipid classes were resolved by t.l.c. or h.p.l.c. as described previously [9]. Subclasses of PC and phosphatidylethanolamine (PE) were resolved by t.l.c. after derivatization to 1,2-diradyl-3-benzoylglycerols and quantified using the method of Blank *et al.* [27]. Purified PC and PE were gently shaken with 30 units of phospholipase C in 1 ml of 0.1 M-Tris/HCl (pH 7.2) and 3 ml of diethyl ether for 3 h at room temperature. After evaporation of the diethyl ether with a stream of N_2 , the diradylglycerols were extracted as described previously [9], dried under N_2 and benzoylated immediately by adding 10 mg of benzoic acid and 4 mg of dimethylaminopyridine in 0.3 ml of benzene and incubating under N_2 at room temperature for 60 min with gentle shaking. The samples were placed in an ice bath and 2 ml of 0.2 M-NaOH was added with shaking at 4 °C. The diradylglycerobenzoates were extracted three times with hexane. The three subclasses (1-alkyl, 1-alkenyl and 1-acyl) of diradylglycerobenzoates were separated by t.l.c. on precoated LK6F plates (Whatman). After development in a solvent system of benzene/hexane/diethyl ether (50:45:4, by vol.) the three lipid subclasses were visualized by u.v. light, iodine vapour or autoradiography, and then scraped. The benzoates were identified by comparison with standards and were well separated with relative R_f values of approx. 0.74, approx. 0.65 and approx. 0.48 for the alk-1-enylacyl, alkylacyl, and diacyl subclasses, respectively.

The relative amounts of diacyl, alkylacyl, and alk-1-enylacyl subclasses which were measured by molar absorbance at 230 nm [27] were 87.5 ± 3.1 (mean $\pm$ s.d., $n = 3$), 4.9 ± 0.5 , and 7.5 ± 1.6 mol % for PC, whereas the PE consisted of 50.9 ± 2.3 , 5.1 ± 0.5 , and 43.9 ± 3.2 mol %, respectively. These values are compatible with the data reported by Blank *et al.* [28].

Protein measurement

Protein was assayed using the method of Lowry *et al.* [29].

Statistical analysis

Data were analysed using Student's *t* test and the

Table 1. Distribution of radiolabelled EPFAs after pulse-labelling of human endothelial cells

Confluent monolayers of HEC were individually pulse-labelled for 15 min with 200 nM ^{14}C -labelled EPFAs, washed and then reincubated for 0, 1 h and 6 h at 37 °C as described in the Experimental procedures section. After counting the medium- and cell-associated radioactivities separately, the cell-associated lipids were extracted and resolved by t.l.c. The data are presented as percentage of total radioactivity. FFAs (cell), cell-associated free fatty acids; FFAs (medium), free fatty acids in the medium.

EPFA ...	Distribution (%)								
	^[14C] AA			^[14C] EPA			^[14C] ETA		
	Time (h)...	0	1	6	0	1	6	0	1
Triacylglycerols	7.4 ± 0.5	9.7 ± 2.7	6.7 ± 1.9	12.4 ± 1.1	14.2 ± 0.9	11.5 ± 1.5	11.0 ± 0.5	11.3 ± 0.8	11.0 ± 1.2
Diacylglycerols	1.9 ± 0.3	2.3 ± 0.5	2.4 ± 1.5	1.2 ± 0.8	1.2 ± 0.9	1.3 ± 0.2	3.2 ± 1.6	1.9 ± 1.2	2.0 ± 1.6
FFAs (cell)	3.9 ± 1.0	3.1 ± 1.1	3.3 ± 1.3	2.4 ± 1.8	2.1 ± 1.1	2.2 ± 0.9	2.1 ± 1.3	2.7 ± 1.2	2.6 ± 1.0
Phospholipids	73.1 ± 1.1	69.1 ± 5.6	67.9 ± 4.4	74.1 ± 2.1	72.7 ± 4.7	63.7 ± 0.5	72.5 ± 3.5	74.2 ± 6.3	61.6 ± 6.3
FFAs (medium)	12.9 ± 4.5	13.5 ± 6.6	19.7 ± 2.1	10.1 ± 3.1	9.9 ± 2.8	21.4 ± 1.0	11.2 ± 2.5	10.0 ± 3.3	22.8 ± 2.6

correlation coefficient r . Linear regression was plotted by the method of least squares.

RESULTS

Distribution of radiolabelled eicosanoid precursor fatty acids in neutral lipids and phospholipids after pulse-labelling of human endothelial cells

Monolayers of cultured HEC were pulse-labelled with EPFAs, washed, and then incubated for up to 6 h. The distribution of radioactivity at various times of reincubation was analysed as described in the Experimental procedures section (Table 1). After washing (time 0), only 2–4 % of the total cell-associated radioactivity is represented by free EPFA. The distribution pattern of radioactivity in each lipid fraction is essentially the same for each of the three EPFAs during 6 h of reincubation following pulse-labelling. Approx. 10–15 % of the total radioactivity is present in the neutral lipid (NL) fraction and this remains constant for up to 6 h. More than 70 % of total radioactivity is present in the phospholipid fraction at time 0. This radioactivity in the phospholipid fraction decreases significantly, while the radioactivity in the free fatty acid (FFA) fraction of the medium increases significantly over 6 h after reincubation of pulse-labelled HEC. The sum of the radioactivities in the phospholipid fraction and the FFA fraction of the media remains unchanged during the 6 h of reincubation. This indicates that there is a spontaneous and significant ($P < 0.01$) release of free EPFAs into the media from phospholipid pools over the 6 h period of reincubation.

Redistribution of radiolabelled eicosanoid precursor fatty acids in glycerophospholipid classes of human endothelial cells

Since there is a significant decrease of radioactivity of total glycerophospholipids labelled by EPFA, the distribution of radiolabelled glycerophospholipid classes was analysed under the same experimental conditions. As shown in Fig. 1, each EPFA-labelled glycerophospholipid shows a different pattern of redistribution during the 6 h of reincubation following pulse-labelling. In the cases of AA and eicosapentaenoic acid ($\omega-3$) (EPA), there is a progressive decrease in PC radioactivity with a concomitant increase in PE radioactivity. The

radiolabelled EPA-esterified PE increases more than does the AA-labelled PE. In contrast, although eicosatrienoic acid ($\omega-6$) (ETA)-labelled PC decreases progressively to the same extent as does AA- or EPA-labelled PC, ETA-labelled PE does not increase and actually decreases slightly. The amount of radiolabelled phosphatidylserine (PS) is much smaller than that of the other glycerophospholipids and does not change significantly over 6 h. Radiolabelled phosphatidylinositol (PI) decreases very slightly. The extent of decrease in radiolabelled PI is much less than that of the decrease in radiolabelled PC and the increase of AA- or EPA-labelled PE.

To examine whether this redistribution of radiolabelled glycerophospholipids, particularly the increases of AA-

Fig. 1. Redistribution of incorporated radioactive EPFAs among the phospholipid classes of human endothelial cells

Confluent cultures of HEC were pulse-incubated with 200 nM of $[^{14}\text{C}]\text{AA}$ ($\square$), $[^{14}\text{C}]\text{EPA}$ ($\square$), or $[^{14}\text{C}]\text{ETA}$ ($\blacksquare$), for 15 min, washed and reincubated at 37 °C for up to 6 h. Phospholipids were extracted at each time point indicated and resolved by t.l.c. or h.p.l.c. Each column represents the mean $\pm$ S.D. for three separate experiments.

or EPA-labelled PE, are influenced by the presence of exogenous EPFA, changes in the distribution of radiolabelled glycerophospholipids was also studied in the presence of exogenous EPFA. The corresponding non-radioactive EPFA at concentrations up to 400 nM were added to the HEC after pulse-labelling with each EPFA and washing the monolayers. The distribution changes of radiolabelled glycerophospholipids are not affected by the presence of an excess of exogenous unlabelled EPFA (results not shown).

Redistribution of eicosanoid precursor fatty acids between phosphatidylcholine and phosphatidylethanolamine subclasses

Under the same experimental conditions as described in Fig. 1, the PC and PE fractions were further resolved into subclasses after conversion into diradylglycerobenzoates. As shown in Fig. 2, more than 95% of the total PC radioactivity is initially present in diacyl PC following pulse-labelling with each of the three EPFAs. Over the 6 h reincubation period, there is a significant ($P < 0.001$ at 1 h reincubation time) and progressive decrease in [^{14}C]EPFAs in diacyl PC. Alkyl PC is more labelled with [^{14}C]AA than with [^{14}C]EPA or [^{14}C]ETA at time 0. This alkyl PC labelled with [^{14}C]AA increases significantly ($P < 0.001$) at 1 h and 6 h after reincubation. In contrast, there is no significant change in [^{14}C]EPA-labelled alkyl PC during 6 h reincubation. The amount of radiolabelled plasmalogen (alkenylacyl) PC is very small and there is no significant change in the ^{14}C radioactivity over time. The relative specific radioactivity of each EPFA-labelled PC subclass was obtained by dividing the amount of each radioactive PC subclass in Fig. 2 by the relative amount of PC subclass presented in the Experimental procedures section. The relative specific radioactivity of AA-labelled alkyl PC, which was smaller

Fig. 2. Redistribution of incorporated radioactive EPFAs among subclasses of phosphatidylcholine

Phosphatidylcholine was resolved as in Fig. 1, and subsequently converted to diradylglycerobenzoates by phospholipase C treatment followed by benzoylation. Diradylglycerobenzoates were separated by t.l.c. Each column represents the mean $\pm$ S.D. for three separate experiments.

Fig. 3. Redistribution of incorporated radioactive EPFAs among subclasses of phosphatidylethanolamine

Phosphatidylethanolamine was resolved and converted to diradylglycerobenzoates, as described for PC in Fig. 2. Diradylglycerobenzoates were separated by t.l.c. Each column represents the mean $\pm$ S.D. for three separate experiments.

than that of EPFA-labelled diacyl PC at time 0, increased about 2-fold and became the highest among those of any of the radiolabelled PC subclasses during 1 h of reincubation. This high relative specific radioactivity of AA-labelled alkyl PC was maintained over 6 h of reincubation. In contrast, none of the other EPFA-labelled PC subclasses continued to demonstrate increased specific radioactivity over 6 h of reincubation (results not shown). The radiolabelled PE subclasses show different patterns of change from the PC subclasses (Fig. 3). Initially >75% of the total PE radioactivity is present in diacyl PE. As opposed to radiolabelled diacyl PC, diacyl PE radiolabelled with either [^{14}C]AA or [^{14}C]EPA tends to increase (without statistical significance) rather than decrease over a 6 h reincubation period. In contrast, diacyl PE radiolabelled with [^{14}C]ETA tends to decrease.

The most striking changes are found in plasmalogen PE. Plasmalogen PE is preferentially labelled with EPA (compared with AA and ETA) at time 0 after pulse-labelling. Plasmalogen PE radiolabelled with either [^{14}C]AA or [^{14}C]EPA increases significantly ($P < 0.001$) during the 6 h reincubation. The time-dependent increase in [^{14}C]EPA-labelled plasmalogen PE is particularly striking (Fig. 3).

Effects of phorbol esters and ionophore A23187 on eicosanoid precursor fatty acid-labelled phospholipids

When phorbol 12-myristate 13-acetate (PMA, 100 nM) is added to monolayers of pulse-labelled HEC pretreated with aspirin, it does not stimulate the release of free EPFAs and does not alter the content of [^{14}C]EPFAs in phospholipids (Fig. 4). The addition of the ionophore A23187 (5 μM) to the monolayers under these same conditions produces an increase in free [^{14}C]AA and a concomitant and equivalent decrease in the [^{14}C]arachidonoyl phospholipids, especially PC. How-

Fig. 4. Distribution change of lipid fractions radiolabelled with EPFAs following addition of A23187 and/or PMA

After pulse-labelling of aspirin pretreated HEC with [14 C]AA, [14 C]ETA or [14 C]EPA, HEC were stimulated with 100 nM-PMA for 10 min (■), 5 μ M-A23187 for 5 min (▨), or 5 μ M-A23187 for 5 min after addition of 100 nM-PMA for 5 min (□), at 37 °C. Changes in the distribution of radioactivity in lipid fractions are indicated as the differences in the percentage distribution before and after stimulation ($\Delta\%$ d.p.m.). Each column represents the mean $\pm$ S.D. for three separate experiments.

ever, [14 C]ETA- or [14 C]EPA-prelabelled monolayers of HEC release much less of those FFAs than do [14 C]AA-prelabelled cells in response to A23187, with an equivalent loss of radiolabelled phospholipids found mostly in PC. When the monolayers of HEC are pretreated for 5 min with the same concentration (100 nM) of PMA that is by itself unable to mobilize FFAs, the release of free [14 C]AA and the decrease of [14 C]AA-labelled PC in response to 5 μ M-A23187 are enhanced approx. 2-fold over that observed without PMA pretreatment. Interestingly, [14 C]AA-labelled PE stimulated with A23187 gains about two times more radioactivity with PMA pretreatment than without it. The release of free [14 C]ETA or [14 C]EPA and the concomitant decrease of PC radiolabelled with the corresponding EPFAs are also enhanced to a lesser extent by pretreatment with PMA; in fact, the magnitude of these changes is much smaller than that induced in [14 C]AA radioactivity by stimulation with A23187 alone.

Although a concentration of 2 μ M-A23187 has no effect on either the release of [14 C]AA into the medium or changes in radiolabelled phospholipid subclasses under these conditions, pretreatment of the HEC with 100 nM-PMA potentiates the effect of 2 μ M-A23187 (results not shown). The magnitude of the response to 100 nM-PMA

Fig. 5. Distribution changes in subclasses of phosphatidylethanolamine and phosphatidylcholine radiolabelled with arachidonic acid following addition of A23187 and/or PMA

Confluent cultures of HEC were stimulated with 100 nM-PMA for 10 min (■), 5 μ M-A23187 for 5 min (▨), or 5 μ M-A23187 for 5 min after addition of 100 nM-PMA for 5 min (□), as in Fig. 4. Changes in the distribution of radioactivity in lipid fractions are indicated as the differences in the percentage distribution before and after stimulation ($\Delta\%$ d.p.m.). Each column represents the mean $\pm$ S.D. for three separate experiments.

plus 2 μ M-A23187 is almost equivalent to that of 5 μ M-A23187 alone with respect to [14 C]AA release and changes in radiolabelled phospholipid subclasses.

Since the most striking findings are obtained with [14 C]AA-labelled PC and PE, those subclasses were further analysed (Fig. 5). The major component contributing to the decrease in the radioactivity of PC is the hydrolysis of diacyl PC. There is no apparent decrease in the plasmalogen PC and a slight increase in the level of alkyl PC. The changes in radioactivity stimulated by ionophore A23187 alone are enhanced by pretreatment with PMA. The increase in the radioactivity of total PE which is described in Fig. 4 is mostly attributable to the increase in [14 C]AA-labelled plasmalogen PE. Pretreatment with PMA results in an approx. 2-fold enhancement of increase in the [14 C]AA-labelled plasmalogen PE and alkyl PC.

Relationship between the amount of released [14 C]arachidonic acid radioactivity and the change of radiolabelled phospholipid subclasses

Since the released [14 C]AA radioactivity is mostly derived from radiolabelled diacyl PC, with a concomitant slight increase in the level of radiolabelling of ether-linked phospholipid subclasses, these relationships were

analysed. When the [^{14}C]AA-labelled HEC were stimulated with either 2 μM or 5 μM -ionophore A23187 with or without pretreatment with 100 nM-PMA, and the distribution of radioactivity in each lipid fraction including the media was studied and expressed as the percentage of total radioactivity, it was found that there is a negative and significant ($r = -0.94$, $P < 0.01$) correlation between the amount of released [^{14}C]AA and that of radiolabelled diacyl PC following stimulation with ionophore A23187 either with or without PMA. Under the same conditions, the amount of radiolabelled plasmalogen PE correlated significantly negatively with that of radiolabelled diacyl PC ($r = -0.72$, $P < 0.05$) and positively with that of released [^{14}C]AA ($r = 0.81$, $P < 0.01$) (results not shown).

DISCUSSION

This report describes the results of studies designed to compare the EPFA-specific remodelling of phospholipid pools of cultured vascular endothelial cells with and without stimulation. The salient new findings are as follows. First, a selective remodelling system is operative among EPFAs between PC and PE and specifically from diacyl to ether-linked glycerophospholipid subclasses. Second, this EPFA-selective remodelling system occurs both in resting cells as well as in activated cells. Third, AA is selectively released upon stimulation by calcium ionophore from diacyl PC pools equally radiolabelled with various EPFAs. The pretreatment of endothelial cells with the protein kinase C activator PMA enhances not only the selective release of AA from diacyl PC but also the specific increase in AA labelling of alkyl PC and plasmalogen PE following stimulation with calcium ionophore. Finally, a close relationship can be established between the amount and selectivity of the released EPFAs from diacyl PC and the increase of radiolabelled EPFAs esterified into plasmalogen PE following stimulation with calcium ionophore both with and without PMA pretreatment.

The remodelling or transesterification of AA into PE and alkyl PC without cell activation has been previously reported in lymphocytes [30], macrophages [30], neutrophils [15], and also endothelial cells [16,31]. It has been recently suggested that the time-dependent transfer of EPFAs from PC to PE may represent a mechanism for the internalization of AA and other polyunsaturated fatty acids, since the membrane phospholipids of endothelial cells and other cells are distributed asymmetrically, with preferential localization of PE on the cytoplasmic surface of plasma membranes [32]. Although specificity of remodelling for AA in comparison with other fatty acids has been suggested in studies of crude enzyme preparations obtained from platelets [10,33] and macrophages [12,13], we have now demonstrated a selective remodelling system in intact cells derived from cultured vascular endothelium. In the case of EPA-labelled unactivated HEC, our data suggesting the presence of a remodelling system from diacyl PC to PE is quite clear: the changes in radiolabelled PI and PS are so small that the increase in radiolabelled PE is explained largely by a loss of radiolabelled diacyl PC. In contrast, in the case of AA-labelled resting HEC, a significant increase of radiolabelled alkyl PC is found during reincubation. Since there is no significant difference in the degree of loss of radiolabelled diacyl PC

among the EPFAs after pulse-labelling of HEC, the differences in changes in radiolabelled PE and alkyl PC among these EPFAs indicate that the phospholipid remodelling system in unactivated endothelial cells possesses selectivity even among the C_{20} fatty acids which differ from each other only in the number and position of double bonds. It has been reported that the number of double bonds in polyunsaturated fatty acids is critical for acylation into platelet phospholipids [34,35]. The lack of remodelling of ETA may be related to the absence of one double bond.

Since the biological functions of this highly selective remodelling system are not clear, it was of great interest to compare the remodelling of EPFAs among phospholipid subclasses in resting and activated cells. We activated the endothelial cells with the ionophore A23187 in order to stimulate phospholipase A_2 rather than phospholipase C [18,37–39], and followed the hydrolysis of PC. It has been found in human platelets that the phorbol ester PMA, which activates protein kinase C, potentiates the ionophore A23187-induced phospholipase A_2 -mediated release of AA, which is derived mainly from PC; PMA alone was found to be incapable of stimulating the release of AA [39]. Our results in HEC are consistent with these findings and extend the analysis to the specific phospholipid subclasses that are radiolabelled by the different EPFAs. It is notable that the specific increase in AA labelling of plasmalogen PE and alkyl PC and its enhancement by PMA pretreatment occurred with a concomitant decrease in labelled diacyl PC only when AA was released.

The increase in AA labelling of alkyl PC and plasmalogen PE during reincubation or upon activation of endothelial cells may occur by several mechanisms. First, trace amounts of free AA may be reacylated by the combined actions of acyl-CoA synthetase and acyl-transferase [2,12,43]. Another mechanism would involve the action of either CoA-dependent or CoA-independent transacylases [10–13,30]. We and others have previously shown [9,36] that, during short-term radiolabelling, free EPFAs are mostly incorporated into PC, and AA is preferentially incorporated into PI, while PE is less readily labelled. We have also shown in the present study that the remodelling of EPFAs during reincubation is not significantly affected by the presence of an excess of non-radioactive EPFAs exogenously added to the media. For these reasons, a transacylase system may be the major mechanism responsible for the appearance of labelled AA in ether-linked glycerophospholipids [2,43], although we cannot exclude that deacylation–reacylation may also be operative. We have found that not only radiolabelled plasmalogen PE but also radiolabelled diacyl PE are increased during reincubation of unactivated endothelial cells following pulse-labelling with AA or EPA, a finding similar to that previously reported [16]. These results suggest that the remodelling processes of EPFAs in resting cells may involve more complex mechanisms than those in activated cells.

It has not been our intent to elucidate the general mechanism of AA release from various membrane phospholipids, since we recognize that problems with interpretation are likely to arise from data obtained by prelabelling with radioactive AA, which is known to be heterogeneously incorporated into the individual molecular species of the various phospholipid classes [40,41]. However, our study design does make it possible

to elucidate a mechanism whereby radioactive AA is selectively released from diacyl PC, since >60% of the total radioactivity in phospholipids was present in diacyl PC, there were no significant differences between the amounts of diacyl PC radiolabelled with AA, ETA and EPA, and a selective release of radioactive AA was noted with a concomitant and equivalent decrease in AA-labelled diacyl PC. Others have previously proposed that the liberation of AA from plasmalogen PE as well as the transfer of AA from PC to plasmalogen PE may be promoted upon cell activation as a result of phospholipase A₂ and transacylase acting in concert [10,42]. Another hypothesis has been proposed that alkyl PC might serve as a precursor for both phospholipase A₂ to release AA selectively, which is transacylated from diacyl PC, and also for PAF production [15]. It has been recently reported that a phospholipase A₂ inhibitory protein phosphorylated by the action of protein kinase C loses its anti-phospholipase A₂ activity [44]. Thus a proposed mechanism for protein kinase C-stimulated cell activation is through its inhibitory action on an endogenous regulator of phospholipase A₂, thereby promoting the mobilization of AA. Our data presented here are consistent with these previous hypotheses to explain the selective release of AA from diacyl PC, supporting the model that AA may be selectively released through an AA-specific transesterification system from diacyl PC to plasmalogen PE and alkyl PC, which is then acted upon by an ether-lipid-specific phospholipase A₂ [17]. In fact, it has been recently reported that both phospholipase A₂-like activity and protein kinase C activity appear to be responsible for the mobilization of AA and lyso PAF from alkyl PC [45]. It has also been demonstrated that porcine aortic endothelial cells release AA from plasmalogen PE with a concomitant decrease of diacyl PC [46]. Although we observed the selective release of radioactive AA from diacyl PC, our findings cannot exclude the possibility of release of EPA and ETA from membrane phospholipids upon cell activation. Since the amount of AA in the phospholipids of HEC is much greater than that of EPA and ETA [28], the liberation of EPA and ETA might be different if greater than trace amounts were incorporated. In fact, Mahadevappa & Holub have reported thrombin-dependent loss of both AA and EPA from the phospholipids (including plasmalogen PE) of platelets of human subjects ingesting a fish lipid concentrate [47].

In summary, cultured human endothelial cells incorporate EPFAs (AA, ETA, EPA) equally into diacyl PC. Upon stimulation, the cells selectively release AA esterified into diacyl PC, with a concomitant increase in AA labelling of plasmalogen PE and alkyl PC. In the resting state, ether-linked glycerophospholipids appear to collect AA to ensure enrichment of AA. Our findings about the remodelling of EPA suggest another important function of dietary fish oil, in that EPA might be able to interfere with the enrichment of AA in ether-linked glycerophospholipids and with the selective release of AA.

This work was supported by National Institutes of Health Grants HL-36028 and HL-27465. H.T. is a recipient of an American Heart Association, Massachusetts Affiliate Fellowship. M.H.K. is the recipient of National Institutes of Health Individual Research Service Award HL-07608. A.I.S. is an Established Investigator of the American Heart Association.

REFERENCES

- Samuelsson, B. (1983) *Science* **220**, 568–575
- Irvine, R. F. (1982) *Biochem. J.* **204**, 3–16
- Sugiura, T., Nakajima, M., Sekiguchi, N., Nakagawa, Y. & Waku, K. (1983) *Lipids* **18**, 125–129
- Mueller, H. W., Purdon, A. W., Smith, J. B. & Wykle, R. L. (1983) *Lipids* **18**, 814–819
- Rosenthal, M. D. (1987) *Biochim. Biophys. Acta* **917**, 279–289
- Wilson, D. B., Prescott, S. M. & Majerus, P. W. (1982) *J. Biol. Chem.* **257**, 3510–3515
- Neufeld, E. J., Wilson, D. B., Sprecher, H. & Majerus, P. W. (1983) *J. Clin. Invest.* **72**, 214–220
- Laposata, M., Reich, E. L. & Majerus, P. W. (1985) *J. Biol. Chem.* **260**, 11016–11020
- Takayama, H., Gimbrone, M. A. Jr. & Schafer, A. I. (1987) *Biochim. Biophys. Acta* **922**, 314–322
- Kramer, R. M. & Deykin, D. (1983) *J. Biol. Chem.* **258**, 13806–13811
- Colard, O., Breton, M. & Bereziat, G. (1984) *Biochem. J.* **222**, 657–662
- Robinson, M., Blank, M. L. & Snyder, F. (1985) *J. Biol. Chem.* **260**, 7889–7895
- Sugiura, T., Masuzawa, Y., Nakagawa, Y. & Waku, K. (1987) *J. Biol. Chem.* **262**, 1199–1205
- Purdon, A. D. & Smith, J. B. (1985) *J. Biol. Chem.* **260**, 12700–12704
- Chilton, F. H. & Murphy, R. C. (1986) *J. Biol. Chem.* **261**, 7771–7777
- Wey, H. E., Jakubowski, J. A. & Deykin, D. (1986) *Biochim. Biophys. Acta* **878**, 380–386
- Loeb, L. A. & Gross, R. W. (1986) *J. Biol. Chem.* **261**, 10467–10470
- Broekman, M. J., Ward, J. W. & Marcus, A. J. (1980) *J. Clin. Invest.* **66**, 275–283
- Neufeld, E. J. & Majerus, P. W. (1983) *J. Biol. Chem.* **258**, 2461–2467
- Broekman, M. J. (1986) *J. Lipid Res.* **27**, 884–891
- Moncada, S. (1982) *Arteriosclerosis* **2**, 193–207
- Takayama, H., Gimbrone, M. A., Jr. & Schafer, A. I. (1987) *Thromb. Res.* **45**, 803–816
- Prescott, S. M., Zimmerman, G. A. & McIntyre, T. M. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3534–3538
- Camussi, G., Aglietta, M., Malavasi, F., Tetta, C., Piacibello, W., Sanavio, F. & Bussolino, F. (1983) *J. Immunol.* **131**, 2397–2403
- Gimbrone, M. A., Jr. (1976) *Prog. Hemostasis Thromb.* **3**, 1–28
- Gimbrone, M. A. Jr., Shefton, E. J. & Cruise, S. A. (1978) *Tissue Cult. Assoc. Man.* **4**, 813–817
- Blank, M. L., Robinson, M., Fitzgerald, V. & Snyder, F. (1984) *J. Chromatogr.* **298**, 473–482
- Blank, M. L., Spector, A. A., Kaduce, T. L. & Snyder, F. (1986) *Biochim. Biophys. Acta* **877**, 211–215
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Trotter, J., Flesch, I., Schmidt, B. & Ferber, E. (1982) *J. Biol. Chem.* **257**, 1816–1823
- Sivarajan, M., Hall, E. R., Wu, K. K., Rafelson, M. E. & Manner, C. (1984) *Biochim. Biophys. Acta* **795**, 271–276
- Hall, E. R., Manner, C. E., Carinhas, J., Snopko, R. & Rafelson, M. (1987) *Biochem. J.* **245**, 151–157
- Kramer, R., Patton, G. M., Pritzker, C. R. & Deykin, D. (1984) *J. Biol. Chem.* **259**, 13316–13320
- Neufeld, E. J., Sprecher, H., Evans, R. W. & Majerus, P. W. (1984) *J. Lipid Res.* **25**, 288–293
- Hajarine, M. & Lagarde, M. (1986) *Biochim. Biophys. Acta* **877**, 299–304

36. Rittenhouse-Simmons, S. & Deykin, D. (1981) in *Platelets in Biology and Pathology 2* (Gordon, J. L., ed.), pp. 349–372, Elsevier/North-Holland Biomedical Press, Amsterdam
37. Rittenhouse, S. & Horne, W. C. (1984) *Biochem. Biophys. Res. Commun.* **123**, 393–397
38. Derksen, A. & Cohen, P. (1975) *J. Biol. Chem.* **250**, 9342–9347
39. Halenda, S. P., Zavoico, G. B. & Fernstein, M. B. (1985) *J. Biol. Chem.* **260**, 12484–12491
40. Mahadevappa, V. G. & Holub, B. J. (1984) *J. Biol. Chem.* **259**, 9369–9373
41. Takamura, H., Narita, H., Park, H. J., Tanaka, K., Matsuura, T. & Kito, M. (1987) *J. Biol. Chem.* **262**, 2262–2269
42. Rittenhouse-Simmons, S., Russell, F. A. & Deykin, D. (1977) *Biochim. Biophys. Acta* **488**, 370–380
43. Lands, W. E. M. (1965) *Annu. Rev. Biochem.* **34**, 313–346
44. Touqui, L., Rothhut, B., Shaw, A. M., Fradin, A., Vargaftig, B. B. & Russo-Marie, F. (1986) *Nature (London)* **321**, 177–180
45. McIntyre, T. M., Reinhold, S. L., Prescott, S. M. & Zimmerman, G. A. (1987) *J. Biol. Chem.* **262**, 15370–15376
46. Brown, M. L., Jakubowski, J. A., Leventis, L. L. & Deykin, D. (1987) *Biochim. Biophys. Acta* **921**, 159–166
47. Mahadevappa, V. G. & Holub, B. J. (1987) *J. Lipid Res.* **28**, 1275–1280

Received 13 May 1988/29 September 1988; accepted 5 October 1988