Selective internalization of arachidonic acid by endothelial cells

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The asymmetric distribution of phospholipids in bovine endothelial-cell membranes was probed with 2,4,6-trinitrobenzenesulphonate and purified phospholipase A₂. The data suggest that phosphatidylethanolamine is primarily located in the inner lipid bilayer, as reported for other cell types. Stearic acid is taken up by the endothelial cells and is randomly distributed among the membrane phospholipids. In contrast, the polyunsaturated fatty acids (arachidonic, eicosatrienoic and eicosapentaenoic acids) have initial incorporation into the phosphatidylcholine fraction. These fatty acids then undergo a time-dependent transfer from phosphatidylcholine to phosphatidylethanolamine. Thus we propose that endothelial cells possess a mechanism for the selective internalization of polyunsaturated fatty acids.

INTRODUCTION

Endothelial-cell eicosanoid biosynthesis, especially that of prostacyclin, is thought to play an important role in vascular homeostasis because of its ability to inhibit platelet aggregation and relax smooth muscle. The three fatty acids which can serve as precursors for the biosynthesis of eicosanoids are eicosatrienoic (ETA; C_{20:3}^{ω6}), arachidonic acid (AA; C_{20:4}^{ω6}) and eicosapentaenoic acid (EPA; C_{20:5}^{ω3}). In intact cells a major determinant of eicosanoid synthesis is the availability of non-esterified fatty acid, which is governed by both the level of fatty acid esterified to phospholipids and the activities of the cell's phospholipases [1,2].

The incorporation and distribution of AA into a number of cells, including endothelial cells, has been reported [3–6]. Arachidonate is rapidly incorporated into both the phospholipids and TGs. Reports on the incorporation of AA into the TGs of endothelial cells have varied considerably and probably reflect differences in both the concentration of AA used and the methodology employed [3–7]. Denning *et al.* [4] have shown that AA incorporation into the TGs is concentration-dependent, such that, at AA concentrations of ≥ 50 μM, more AA is incorporated into the TGs than into the phospholipids. Furthermore, Sivarajan *et al.* [3] have demonstrated that the incorporation of AA into endothelial-cell TGs is time-dependent. Substantial quantities of AA are rapidly esterified to the TGs that are subsequently released and incorporated into the membrane phospholipids. Studies on the incorporation of ETA, AA and EPA into the cell lipids of skin fibroblasts and keratinocytes [8,9] indicate that ETA and EPA are as effectively incorporated into TGs as is AA. Sicard & Lagarde [10] have also observed a significant incorporation of ETA and EPA into endothelial cell TGs.

Recent studies [3,11] on the uptake and distribution of AA into endothelial-cell phospholipids have reported high initial incorporation of radiolabelled AA into PC, followed by a partial transfer of that AA to PE over 24 h. Although the distribution patterns of acylated ETA, AA

and EPA differ somewhat among different cell types, in general each of these fatty acids is readily esterified into the phospholipids. However, studies in some cell types (e.g. keratinocytes, platelets and erythrocytes) have reported that EPA is incorporated into PI or PS + PI less effectively than is AA [9,12,13]. On the other hand, other cell types (e.g. endothelial cells, skin fibroblasts and macrophages) appear to incorporate substantial amounts of EPA into all of the phospholipids, including PI and PS [8,14,15]. Whether or not ETA and EPA undergo a time-dependent transfer from PC to PE, as described for AA, has not been investigated. Considering the recent interest in highly-polyunsaturated-fat diets, it is important to understand the nature of the uptake and distribution of these fatty acids.

The internal leaflet of the plasma membrane of erythrocytes and platelets has been reported to be enriched with PE. Thus if AA, ETA and EPA are preferentially transferred to PE with time, then they may also be selectively internalized. The internalization of these fatty acids may be important both in the maintenance of membrane integrity and in providing substrate to prostaglandin H synthase.

The present study was undertaken to answer the following questions: (1) do endothelial cells discriminate between ETA, AA and EPA in their pattern of uptake and distribution? (2) do endothelial cells, like other cells, contain an enrichment of PE in their internal lipid monolayer? (3) do endothelial cells preferentially internalize polyunsaturated fatty acids, as opposed to a random distribution of saturated fatty acids? and (4) is the internalization of polyunsaturated fatty acids an integral function of endothelial cells to be found in cells derived from aortic, venous and microvascular sources?

MATERIALS AND METHODS

All chromatographic solvents were of reagent grade and obtained from commercial sources. Tissue-culture media and supplements were purchased from Gibco,

Abbreviations used: AA, arachidonic acid; BAEC, bovine aortic endothelial cells; BREC, bovine retinal microvascular endothelial cells; BVEC, bovine sphenous-vein endothelial cells; BSA, bovine serum albumin; EPA, eicosapentaenoic acid; ETA, eicosatrienoic acid; PG, prostaglandin; 6OPGF_{1α}, 6-oxoprostaglandin F_{1α}; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PLA₂, phospholipase A₂; PGI₂, prostacyclin; SA, stearic acid; TG(s), triacylglycerol(s); TNBS, 2,4,6-trinitrobenzenesulphonate.

Grand Island, NY, U.S.A. SA, phospholipid standards, PLA₂ and TNBS were obtained from Sigma, St. Louis, MO, U.S.A. AA, ETA and EPA were obtained from Nu-Chek, Elysian, MN, U.S.A. [1-¹⁴C]SA (> 50 mCi/mmol) and [1-¹⁴C]AA (> 50 mCi/mmol) were obtained from Amersham Corp., Arlington Heights, IL, U.S.A. [1-¹⁴C]Eicosa-8,11,14-trienoic acid (50 mCi/mmol), [1-¹⁴C]eicosa-5,8,11,14,17-pentaenoic acid (100–500 mCi/mmol) and 6-[5,8,9,11,12,14,15-³H(n)]oxoprostaglandin F_{1α} (120–180 mCi/mmol) were obtained from New England Nuclear, Boston, MA, U.S.A. T.l.c. plates (high-performance silica-gel 60) were obtained from EM Reagents, Darmstadt, Germany.

Endothelial-cell cultures

Bovine endothelial cells were obtained from the aortic arch (BAEC), saphenous vein (BVEC), and retina (BREC) essentially as previously described [16,17]. Endothelial cells, passages 2–5, were grown in RPMI 1640 medium containing heat-inactivated 20% (v/v) fetal-calf serum, Hepes buffer (15 mM), penicillin (50 units/ml), streptomycin (50 µg/ml), neomycin (100 µg/ml) and L-glutamine (2 mM). Each data point represents results from cells derived from at least three aortas, two saphenous veins or three preparations of retinal microvascular cells. All three cell types (i.e. BAEC, BVEC and BREC) appeared as essentially homogeneous populations of uniformly sized, tightly packed, polygonal cells. In addition, each of the cell types exhibited Factor VIII antigen, angiotensin-converting-enzyme activity and the ability to synthesize increased quantities of PGI₂ when stimulated with bradykinin.

Labelling cell lipids

Before labelling, the endothelial cells were washed overnight with serum-free media and once more the following morning for 2 h. Cells were then incubated with serum-free media supplemented with radiolabelled AA. [1-¹⁴C]AA and unlabelled AA were added as the sodium salt to serum-free media to achieve final concentrations of 1–100 µM containing (2–3) × 10⁵ d.p.m./4 ml. The cells were initially incubated with 4 ml of AA-enriched media (10 µM) at 37 °C for various time periods from 5 min to 48 h to determine their rate of AA uptake. Similar experiments were performed with media enriched with SA, ETA and EPA. In addition to the initial uptake experiments, the response of endothelial cells to 15 min incubations with different concentrations of ETA, AA and EPA was investigated. The temporal distribution of these fatty acids in endothelial-cell lipids was assessed by pulse-labelling the cells with the described 10 µM-1-¹⁴C-labelled-fatty-acid-enriched media. In these experiments the cells were incubated with the radiolabelled fatty acid for time periods which allowed for approx. 50% uptake of the radiolabel (i.e. 30 min for ETA, AA or EPA, and 3 h for SA). After this labelled pulse, the cells were washed twice in serum-free media and incubated for various times with the corresponding unlabelled fatty acid at a concentration of 5 µM.

Lipid extraction and analysis

After incubating the cells with fatty-acid-enriched medium, the medium was decanted and counted for radioactivity. The cells were then rinsed twice with phosphate-buffered saline (6 mM-phosphate buffer con-

taining 0.145 M-NaCl), pH 7.4, scraped from the flask and extracted with chloroform/methanol as described by Folch *et al.* [18] Since less than 1% of the radioactivity remained in the cell debris after extraction, the organic extract was used to determine total incorporated radioactivity. These extracts were evaporated to dryness under N₂ and then resuspended in 100 µl of chloroform/methanol (2:1, v/v). The distribution of radiolabelled fatty acid was determined by t.l.c. on high-performance silica-gel 60 plates. Neutral lipids and non-esterified fatty acids were separated from phospholipids with a solvent system of n-heptane/diethyl ether/methanol/acetic acid (42:10:2:1, by vol.). The phospholipids were further separated in solvent systems containing chloroform/methanol/acetic acid/water (25:15:4:1, by vol.) and chloroform/methanol/formic acid/acetic acid/water (45:10:5:1:1, by vol.) Standards were added to each chromatogram and the lipid spots were revealed by exposure to I₂ vapour. The area corresponding to each standard was removed by scraping, extracted with methanol and counted for radioactivity in a scintillation counter.

Radioimmunoassay for 6OPGF_{1α}

Since prostacyclin is rapidly hydrolysed to 6OPGF_{1α} in aqueous solutions, the prostacyclin production of the endothelial cells was assayed by measuring the 6OPGF_{1α} immunoreactivity in the decanted media. Each sample was run in duplicate at three dilutions, and values were obtained within the linear portion of the standard curve. Antiserum against 6OPGF_{1α} was obtained by the repeated immunization of rabbits with 6OPGF_{1α} coupled to bovine serum albumin. The radioimmunoassay was performed by incubating 0.2 ml of 6OPGF_{1α} standards or samples with 0.1 ml of diluted antiserum (final resultant dilution of 1:800) and 0.1 ml of ³H-labelled 6OPGF_{1α} (10000 c.p.m.). The incubation was carried out at room temperature for 1 h. Separation of the bound and free antigen was achieved with γ-globulin-coated charcoal. The supernatant obtained after centrifugation was counted for radioactivity in a Packard liquid-scintillation counter. The ratio of bound to total radioactivity was calculated for each sample, and the concentration of 6OPGF_{1α} was determined from a standard curve after logit transformation [6]. The antiserum showed little cross-reactivity with PGF_{2α} (3.5%), PGE₂ (0.7%), thromboxane B₂ (0.03%) or 6OPGE₁ (7.7%). The sensitivity of the assay was 15 pg/200 µl (i.e. the concentration of unlabelled 6OPGF_{1α} that can inhibit the binding of [³H]6OPGF_{1α} = 20%).

TNBS labelling of phospholipids

The reactivity of endothelial-cell phospholipids with TNBS was determined by using a suspension of 16 × 10⁶ intact endothelial cells or endothelial cells that had been lysed by sonication. Stock solutions of TNBS [32 mM in 5% (w/v) NaHCO₃] were made fresh daily. The stock solution of TNBS was diluted in the suspension buffer, which contained 5.5 mM-glucose, 0.5% BSA, 40 mM-NaCl, 120 mM-NaHCO₃, to a final TNBS concentration of 1.5 mM [19]. Endothelial-cell suspensions were prepared by treating BAEC monolayers with trypsin for 30 s–1 min with trypsin/EDTA (Gibco; 0.5 g of trypsin and 0.2 g of Na₄EDTA/litre of Hanks balanced salt solution without CaCl₂, MgCl₂·6H₂O and MgSO₄·7H₂O). A portion of the cells was mechanically

disrupted by sonication. Intact and lysed endothelial-cell preparations were pelleted and then resuspended in 30 ml of TNBS containing suspension buffer and incubated for 3 h at room temperature. The reaction was terminated by centrifugation. The intact cells were removed by centrifugation at 2000 *g* for 15 min, whereas the lysed-cell membranes were centrifuged at 103 000 *g* for 1 h. The supernatants were decanted and the pellets washed twice with 20 ml of suspension buffer without TNBS. The final cell pellets were extracted with chloroform/methanol (1:1, v/v). The lipid extracts were filtered, evaporated under N₂ and redissolved in 100 μ l of methanol. T.l.c. of these methanolic solutions was performed on 10 cm $\times$ 10 cm HP-silica-gel-60 plates as described by Schick *et al.* [20]. Briefly, silica-gel plates were spotted and then developed for three-quarters of their length in chloroform/methanol/NH₃ (14:6:1, by vol.). The plates were removed from the solvent, air-dried, and re-developed to their full length in chloroform to separate further the TNBS derivative of PE. PE that had not reacted was revealed with ninhydrin. PE and PS standards were allowed to react with TNBS and chromatographed in order to identify the TNBS-PE and TNBS-PE derivatives formed using the reaction of TNBS with the lysed cells.

Treatment of endothelial cells with PLA₂

Confluent cultures of BAEC were washed for 2 h at 37 °C with RPMI 1640 containing 0.4% delipidated BSA. They were then washed twice with RPMI 1640 (30 min each time). The cells were then labelled with [1-¹⁴C]AA for 2 h as described under 'Labelling cell lipids' above. A minimum of 80% uptake of the radiolabelled arachidonate was observed. After labelling, the cells were washed twice in RPMI 1640/0.4% BSA (30 min each time) to remove any loosely associated labelled AA. Finally the cells were incubated with 20 units Indian cobra (*Naja naja*) venom PLA₂ or 30 units of honey-bee (*Apis mellifera*) venom PLA₂ dissolved in 4 ml of RPMI 1640 containing 0.4% BSA for 1 h at 37 °C. The percentage of [1-¹⁴C]AA released was determined by counting the radioactivity in the cell supernatant. Identical flasks of cells were similarly labelled for 2 h, incubated for an additional 22 h in RPMI 1640 at 37 °C and finally stimulated with PLA₂ as described above. Stock solutions of *Naja naja* and *Apis mellifera* PLA₂ (100 units/ml) were made in glycerol/phosphate-buffered saline (1:1, v/v) and stored at -20 °C until needed, as described by Roelofsen & Zwaal [21].

RESULTS

The incorporation of ETA and EPA by endothelial cells was similar to that of AA. At concentrations of 10 μ M, these fatty acids exhibited equal rates of uptake, with intracellular concentrations reaching similar levels within 2–3 h. Maximum uptake of total label added was 86.8, 80.3 and 74.8% for ETA, AA and EPA respectively. These maxima were not statistically different (Fig. 1). Similar results were obtained with venous- and microvascular-derived endothelial cells, as shown here for the bovine aortic cells. Less than 1% of the added AA was metabolized to 6OPGF_{1 α} as determined by radio-immunoassay. SA, the most prevalent saturated fatty acid in endothelial cells, was taken up much more slowly by the cells than were any of the polyunsaturated fatty

Fig. 1. Rate of incorporation of AA, ETA and EPA into bovine aortic endothelial cells

Cells were incubated at 37 °C with medium supplemented with either 10 μ M-AA ($\square$), -ETA ($\triangle$) or -EPA ($\circ$) from 5 min to 12 h. Each point represents the mean $\pm$ s.d. for duplicates of three experiments.

acids tested. Although this phenomenon has already been described for BAEC cells, we found almost identical results in both BVEC and BREC (Fig. 2).

The extent to which these polyunsaturated fatty acids were taken up as TG or phospholipid was dependent on the total amount of fatty acid incorporated into the endothelial-cell lipids. As their intracellular concentrations increased, ETA, AA and EPA were similarly incorporated into cellular phospholipids, with a maximum level of 6–7 nmol/15 min per 10⁶ cells (Fig. 3). However, ETA was less extensively incorporated into the TGs than was either AA or EPA. The maximal incorporation of AA and EPA into TG was similar (9–10 nmol/15 min per 10⁶ cells), which exceeded their incorporation into the phospholipids (6.5–7.0 nmol/15 min per 10⁶ cells) at high concentrations. Although ETA was not incorporated into the TG fraction as readily as the other two fatty acids, substantial TG labelling was observed at the higher concentrations of ETA (6.8 nmol/15 min per 10⁶ cells). An interesting feature of TG labelling was that it continued to increase even after the phospholipid labelling had reached its maximum level. Once TG labelling approached its maximum, additional intracellular fatty acid remained non-esterified (Fig. 3).

The uptake and redistribution of SA, ETA, AA and EPA into the various lipid fractions of BAEC, BVEC and BREC was further investigated by using the pulse-chase experiments described in the Materials and methods section. Cells that were labelled with [1-¹⁴C]-AA or -EPA for 30 min and then chased with unlabelled fatty acid showed a significant initial esterification to TG, which declined over the following 8 h. This decline in polyunsaturates acylated to TG was accompanied by a reciprocal increase in phospholipid labelling (Fig. 4). SA, unlike the polyunsaturates, remained non-esterified, with a very slow incorporation into the phospholipids, as previously reported [3].

The time-dependent distribution patterns of SA and ETA, AA and EPA into the various phospholipid fractions were significantly different from each other (Figs. 5 and 6).

Fig. 2. Rate of incorporation of AA (●) or SA (■) into bovine endothelial cells

Cells were incubated at 37 °C with medium supplemented with either 10 μ M-AA or 10 μ M-SA from 5 min to 24 h. For each point, a minimum of four flasks were used to calculate the mean $\pm$ S.D.

SA appeared to undergo a random incorporation into all of the phospholipid fractions, with no major change in its pattern of distribution within the 48 h investigated (Fig. 5). AA, on the other hand, was rapidly incorporated into the retinal-cell phospholipids and then underwent a major redistribution from PC to PE. For example, after a 1 h chase, $79.8 \pm 5.0\%$ ($\bar{x} \pm$ S.D) of the phospholipid label was incorporated into the PC fraction and $9.6 \pm 3.4\%$ of the label was in the PE fraction (Fig. 5). After a 48 h chase, only $38.3 \pm 2.0\%$ of the label was still incorporated into the PC fraction, and the PE fraction was enriched with AA such that it now contained $45.5 \pm 6.8\%$ of the phospholipid label. Thus the percentage of phospholipid-associated [14 C]AA was decreased 41.5% in the PC fraction, whereas the percentage of [14 C]AA in PE increased 35.9%. The redistribution of ETA and EPA was also investigated (Fig. 6). EPA ($C_{20:5}$) showed a pattern of redistribution very similar to that observed with AA ($C_{20:4}$). The percentage of phospholipid-associated labelling in PC fell 35.2% between the 1 and 48 h chase points. PE labelling, on the other hand, was increased 30.9% during that same time period. Although ETA ($C_{20:3}$), also showed a significant decrease in PC labelling with time, the decrease in PC labelling was accompanied by an increase in labelling of all the other phospholipid fractions. The significance of these differences is unknown.

The localization of PE in the endothelial-cell membranes was probed with TNBS. Since TNBS cannot cross the cell membrane, its reaction with the free amino groups of PE and PS has been used to measure the accessibility of the polar head groups of these phospholipids. Both intact and lysed endothelial cells were allowed to react with TNBS for 3 h; their phospholipids were then extracted with chloroform/methanol and separated by t.l.c. Visually the chromatogram of the intact-cell phospholipids showed no yellow spot with an R_F value of 0.56 ± 0.02 (the R_F value of the PE standard, that had reacted with TNBS). In contrast, the lysed-cell chromatogram displayed a prominent yellow spot with that R_F value. To confirm this differential TNBS

Fig. 3. Variation of fatty acid distribution with the rate of intracellular incorporation

Bovine aortic endothelial cells were incubated at 37 °C for 15 min with medium labelled with either [14 C]AA ($\square$), [14 C]ETA ($\triangle$) or [14 C]EPA ($\circ$) at various concentrations. The distribution of fatty acid into phospholipids (PL), TGs and non-esterified fatty acid (NFA) was determined by t.l.c. as described in the Materials and methods section. Each point represents the mean $\pm$ S.E.M. for duplicates of four experiments.

derivatization, we measured, in lipid extracts of the reacting cells, the absorbance at 337 nm, the absorption maximum of the TNBS-PE derivative. The 337 nm absorbance of the lysed-cell extract was 1.29 ± 0.56 , that of the intact cells was 0.32 ± 0.09 ($n = 3$). To quantify further the percentage of TNBS-derivatized PE in the cells that had reacted, additional experiments were performed with endothelial cells prelabelled with [14 C]AA for 48 h. The percentage of total radiolabelled PE that had reacted with TNBS was determined by

Fig. 4. Percentage distribution of label into bovine aortic endothelial-cell lipids at various time intervals after a 10 μ M pulse of labelled fatty acid

Pulse-labelling was performed as described in the Materials and methods section with ETA, AA and EPA. The percentage distribution of label in phospholipids (○), TGs (△) and non-esterified fatty acid (□) was determined by t.l.c. Each point represents the mean $\pm$ S.E.M. for duplicates of three experiments.

scraping off and counting for radioactivity both the PE and TNBS-PE spots from the t.l.c. plates. Only 19.3% of the PE-associated radioactivity chromatographed with TNBS-PE in the intact-cell phospholipids, whereas 72.7% of the PE-associated radioactivity in the lysed-cell phospholipids chromatographed with the TNBS-PE standard.

In addition to probing with TNBS, we utilized PLA₂ purified from bee and *Naja naja* venoms to monitor the internalization of [¹⁴C]AA. Intact cells were labelled for 2 h with [¹⁴C]AA. These cells were treated for 1 h with

Fig. 5. Time-dependent changes in AA (a) and SA (b) distribution among bovine retinal endothelial cell phospholipids

The cells were pulsed with 10 μ M-AA or -SA for 30 min or 3 h respectively. The cells were rinsed and then chased for various time intervals with 5 μ M fatty acid. The phospholipids were extracted and separated by t.l.c. and the ¹⁴C content of PC (●), PE (■) and PS+PI (▲) was determined. Each point represents the mean $\pm$ S.D. for a minimum of four flasks.

PLA₂, either immediately after the 2 h labelling or 22 h after the labelling. The percentage of incorporated radioactivity released was determined and compared (Table 1). Cells that were treated with 30 units of bee venom PLA₂ released an average of 3.6% of their incorporated [¹⁴C]AA after 2 h of labelling, which was significantly higher (P 0.04) than the 2.0% of [¹⁴C]AA released from the cells labelled for 24 h. A similar difference in [¹⁴C]AA release was observed when cells were treated with *Naja naja* venom PLA₂ after 2 and 24 h labellings.

DISCUSSION

The polyunsaturated fatty acids (ETA, AA and EPA) are rapidly incorporated into both the phospholipids and TGs of endothelial cells (BAEC, BVEC and BREC). SA was not incorporated into the TGs and was only slowly esterified into the phospholipid fraction. These data extend our previous observations on the distribution of AA in BAECs [3]. Our pulse-chase data suggest that both AA and EPA can be rapidly esterified to TGs and then transferred from TG to phospholipid moieties (Fig. 3). Since endothelial cells must acquire AA and EPA from exogenous sources, incorporation of these acids into TGs may serve as an efficient storage system such that the cells can take advantage of the times when

Fig. 6. Time-dependent changes in ETA (a) and EPA (b) distribution among bovine aortic endothelial-cell phospholipids

The cells were pulsed with 10 μ M-ETA or -EPA for 30 min, washed, and chased with 5 μ M fatty acid for various periods. The 14 C content of PC (●), PE (■) and PS+PI (▲) was determined. A minimum of four flasks were used to calculate the mean $\pm$ S.D. for each point.

Table 1. Release of [14 C]AA by PLA₂ 2 h and 24 h after labelling

PLA ₂ source	Time (h)*	Release of [14 C]AA† (%)
Bee venom (30 units; n = 6)	2	3.64 $\pm$ 0.97
	24	1.95 $\pm$ 1.52§
<i>Naja naja</i> venom (20 units; n = 5)	2	8.25 $\pm$ 3.50
	24	4.14 $\pm$ 0.74§

* The cells were treated with PLA₂ either 2 or 24 h after the [14 C]AA was added to the cells.

† The specific release of 14 C-AA in response to PLA₂ treatment for 1 h at 37 °C.

‡ $\bar{x} \pm$ S.D.

§ $P \leq 0.04$ when compared with the 2 h samples.

surface of plasma membranes, is probably a common feature of plasma membranes [22,23]. The pulse-chase data presented here suggest that, in endothelial cells, polyunsaturated fatty acids (especially AA and EPA) are specifically transferred from PC to PE in a time-dependent manner (Figs. 5 and 6). Although our TNBS data do not provide definitive proof that PE is localized on the internal leaflet of the plasma membrane of endothelial cells, it is consistent with the general observation that there is little PE on the external surface of cells. If most PE is preferentially localized in the endoplasmic-membrane leaflet and there is a time-dependent transfer of [14 C]AA from PC to PE, then one would expect to find a time-dependent internalization of [14 C]AA. In effort to obtain direct evidence for this hypothesis, we treated BAEC cells for 1 h with PLA₂ either 2 h after adding [14 C]AA to the cells or 24 h after adding [14 C]AA. The release of [14 C]AA by PLA₂ was significantly less 24 h after the [14 C]AA was added than at 2 h. A 46% decrease in the release of [14 C]AA was observed when the bee venom enzyme was used, and a 50% decrease was observed with the *Naja naja* venom enzyme. These values suggest that 40–50% less radio-labelled AA was available to exogenously added PLA₂ at 24 h than at 2 h after labelling. Perret and co-workers [24] have used a similar approach to conclude that AA is predominantly localized in the internal leaflet of the platelet plasma membrane.

This time-dependent internalization of AA appears to be an integral function of endothelial cells, since this phenomenon was observed in aortic, venous and retinal-microvascular endothelial cells. To date the mechanism(s) involved in this internalization are unknown. Although our results do not define the reactions involved in this transfer, they do allow us to eliminate a few potential reactions. For instance, if the fatty acid transfer were to occur through a polar-head-group exchange, our pulse-chase experiments would have indicated an increase in SA incorporation into PE. Furthermore, non-specific deacylation-reacylation reactions would lead to a distribution of the polyunsaturated fatty acids among the phospholipid fractions in proportion to their concentrations. However, cultured human umbilical-vein endothelial cells have been reported to contain twice the quantity of AA acylated to PE as to PC, despite the fact that endothelial cells contain approximately twice as much PC as PE [7]. Therefore we propose that the transfer of these polyunsaturated fatty acids from PC to PE is mediated by specific enzymes similar to the phospholipid acyltransferases or the arachidonyltransferases that have been described in platelets [26–28].

The functional significance of the transfer of polyunsaturated fatty acids from PC to PE is unknown. The result of such a specific transfer reaction would be membranes in which the two leaflets of the bilayer have distinctly different physical properties. Indeed one might speculate that the greater 'fluidity' of the inner leaflet that has been proposed may be partially due to its increased concentration of polyunsaturated fatty acids [21].

plasma levels are high and phospholipid stores are saturated.

The asymmetric distribution of phospholipids in the plasma membrane, where the aminophospholipids PS and PE are preferentially located on the cytoplasmic

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