Metabolism of n–3 polyunsaturated fatty acids and modification of phospholipids in cultured rabbit aortic smooth muscle cells

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Abstract The metabolism of the linolenic acid family (n-3) of fatty acids, e.g., linolenic, eicosapentaenoic, and docosahexaenoic acids, in cultured smooth muscle cells from rabbit aorta was compared to the metabolism of linoleic and arachidonic acids. There was a time-dependent uptake of these fatty acids into cells for 16 hr (arachidonic > docosahexaenoic, linoleic, eicosapentaenoic > linolenic), and the acids were incorporated mainly into phospholipids and triglycerides. Eicosapentaenoic and arachidonic acids were incorporated more into phosphatidylethanolamine and phosphatidylinositol plus phosphatidylserine and less into phosphatidylcholine than linolenic and linoleic acids. Docosahexaenoic acid was incorporated into phosphatidylethanolamine more than linolenic and linoleic acids and into phosphatidylinositol plus phosphatidylserine less than eicosapentaenoic and arachidonic acids. Added linolenic acid accumulated mainly in phosphatidylcholine and did not decrease the arachidonic acid content of any phospholipid subfraction. Elongation-desaturation metabolites of linoleic acid did not accumulate. Cells treated with eicosapentaenoic acid accumulated both eicosapentaenoic and docosapentaenoic acids mainly in phosphatidylethanolamine, and the arachidonic acid content was decreased. Added docosahexaenoic acid accumulated mainly in phosphatidylethanolamine and decreased the content of both arachidonic and oleic acids. The following conclusions are drawn from these results. *1*) The three n-3 fatty acids are utilized differently in phospholipids. *2*) The arachidonic acid content of phospholipids is reduced by eicosapentaenoic and docosahexaenoic acids, but not by linolenic acid. *3*) Smooth muscle cells have little or no desaturase activity, but have significant elongation activity for polyunsaturated fatty acids. —**Morisaki, N., T. Kanzaki, Y. Fujiyama, I. Osawa, K. Shirai, N. Matsuoka, Y. Saito, and S. Yoshida.** Metabolism of n-3 polyunsaturated fatty acids and modification of phospholipids in cultured rabbit aortic smooth muscle cells. *J. Lipid Res.* 1985. **26:** 930–939.

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Recently, special attention has been paid to the n-3 family (linolenic family) of fatty acids from the viewpoint of prevention of atherosclerosis. Of these fatty acids eicosapentaenoic acid [20:5(n-3), EPA] has been extensively studied in serum and platelets. Greenland Eskimos, whose diet is rich in EPA (1), have a very low incidence of cardiovascular diseases (2). Serum and platelets of Greenland Eskimos are rich in EPA (3, 4). EPA reduces platelet aggregation in vivo (5–7) and in vitro (8–11) in humans and animals, and the mechanism of its antiaggregatory effect has been studied. EPA reduces the arachidonic acid [20:4(n-6), AA] content of platelet phospholipid (7), resulting in reduced substrate availability for cyclooxygenase to make thromboxane A_2. It also reduces the release of AA from platelet phospholipid (10) and competitively inhibits the conversion of AA to thromboxane A_2 (9).

The effect of EPA on the vascular wall, which is the most important tissue in terms of atherosclerosis, is less certain and still controversial. Studies showed that an EPA-rich diet inhibited PGI_2-like synthesis (12, 13) or stimulated it (6) in the aorta; the mechanism of the stimulatory effect was not clear (6). In human umbilical cord, EPA was found to be converted to PGI_3 (14), which has an effect similar to that of PGI_2 on platelet aggregation. But it was not efficiently converted to PGI_3 in rat aorta (6), cultured aortic smooth muscle cells (15, 16), or cultured human umbilical endothelial cells (17). EPA reduced cholesterol esterase activity in rat aorta in vivo (5), but the mechanism of this effect is not known. Although the phospholipid fatty acid composition of vascular wall cells is very important for these phenomena,

Abbreviations: AA, arachidonic acid [20:4(5,8,11,14)]; EPA, eicosapentaenoic acid, [20:5(5,8,11,14,17)]; DHA, docosahexaenoic acid [22:6(4,7,10,13,16,19)]; LLA, linolenic acid, [18:3(9,12,15)]; LA, linoleic acid, [18:2(9,12)]; LDL, low density lipoprotein; DME, Dulbecco's modified Eagle's medium; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; TLC, thin-layer chromatography.

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little is known about the modification of phospholipids by EPA, especially in smooth muscle cells.

The diet of Greenland Eskimos is also rich in another n-3 fatty acid, docosahexaenoic acid [22:6(n-3), DHA] (1). DHA has recently been reported to have an anti-aggregatory effect like EPA in vivo (5, 7) and in vitro (18). DHA was found to inhibit PGI_2 synthesis in cultured guinea pig smooth muscle cells (16) and human umbilical endothelial cells (17) in vitro, but the metabolism of DHA in the vascular wall is unknown.

Moreover, there is controversy as to whether linolenic acid [18:3(n-3), LLA] has an effect on the vascular wall similar to that of its elongated, desaturated metabolites, EPA and DHA, or whether it is efficiently converted to EPA and DHA in the body or the aorta. Dyerberg, Bang, and Aagaard (19) reported that oral administration of LLA for 7 days to humans was not long enough to cause significant increases in the levels of EPA and DHA in the plasma. Beitz, Mest, and Förster (20) found, however, that LLA administration increased EPA and DHA in human plasma phospholipids. In rats, LLA increased the EPA and DHA contents of the liver and spleen (21), macrophages (22), and platelets and the liver (23). Although LLA did not affect in vitro synthesis of PGI_2 in cultured human umbilical endothelial cells (19), it reduced the in vivo synthesis of PGI_2 in the aorta (24), of PGE_2 in macrophages (22), and of PGE_2 or $PGF_{2\alpha}$ in liver, thymus, spleen, and brain slices (21) from rats. The mechanisms of these phenomena are not known and very little is known about the metabolism of LLA in the vascular wall.

Smooth muscle cells are as important as endothelial cells in the vascular walls because they are one of the types of cells responsible for lipid accumulation and cell proliferation, and thus are closely related to development of atherosclerosis. Furthermore, smooth muscle cells synthesize prostanoids such as PGI_2 and PGE_2 (as reviewed elsewhere (25)). Modification of phospholipids of smooth muscle cells could affect these phenomena.

In this work we have investigated the metabolism of the n-3 family of fatty acids and their effect on phospholipids in cultured rabbit aortic smooth muscle cells.

MATERIALS AND METHODS

Materials

Linoleic acid [18:2(n-6)], linolenic acid [18:3(n-3)], arachidonic acid [20:4(n-6)], and docosahexaenoic acid [22:6(n-3)], all of more than 99% purity, were purchased from Nu-Chek-Prep Co. (Elysian, MN). Eicosapentaenoic acid [20:5(n-3)] (98% pure) was kindly supplied by Nippon Oil and Fat Co. (Tokyo). [1-^{14}C]LA (52.6 mCi/mmol), [1-^{14}C]LLA (51.0 mCi/mmol), [1-^{14}C]AA (56.9 mCi/mmol), [1-^{14}C]EPA (56.3 mCi/mmol), and [U-^{14}C]DHA (160 mCi/mmol) were purchased from New England Nuclear (Boston, MA). Dulbecco's modified Eagle's medium (DME) and fetal bovine serum (Lot 33P7430) were purchased from Grand Island Biological Co. (Grand Island, NY). The lipid content of the serum was as follows: total cholesterol 41 mg/dl, triglyceride 10 mg/dl, phospholipid 57 mg/dl, free fatty acid 223 μEq/l. The fatty acid composition of total lipids was as follows: 14:0, 0.4%; 16:0, 20.4%; 16:1(n-7), 4.2%; 18:0, 17.8%; 18:1(n-9), 25.9%; 18:2(n-6), 3.8%; 18:3(n-3), 0.9%; 20:3(n-6), 2.8%; 20:4(n-6), 8.9%; 20:5(n-3), 0.4%; 22:4(n-6), 1.1%; 22:5(n-3), 4.2%; 22:6(n-3), 4.4%. The fatty acid composition of free fatty acids was as follows: 14:0, 3.7%; 16:0, 27.3%; 16:1(n-7), 5.6%; 18:0, 11.8%; 18:1(n-9), 16.7%; 18:2(n-6), 1.7%; 20:3(n-6), 0.7%; 20:4(n-6), 5.2%; 22:5(n-3), 1.1%; 22:6(n-3), 2.0%. The serum contained 1.8 g/dl albumin.

Tissue culture

Primary cultures of smooth muscle cells were established from the thoracic aorta of a male Japanese white rabbit (*Oryctolagus cuniculus var. domesticus*) by the method of Fischer-Dzoga et al. (26). The thoracic aorta was removed under sterile conditions from rabbits anesthetized with nembutal. The adventitia was carefully removed and the tissues were cut into small pieces (about 2 mm × 2 mm square) and placed in sixteen T-25 flasks (Corning) with 1 ml of medium consisting of DME supplemented with 0.67 mg per ml of sodium bicarbonate, 10 μg per ml of gentamycin, and 10% fetal bovine serum, and incubated at 37°C in a CO_2 (5%) incubator. The medium was renewed once a week. After 2 weeks, when explanted cells had grown out from the explants, the cells were harvested with trypsinization and subcultured in three T-75 flasks (Corning). The following day, the medium was changed to remove the explants and culture was continued with renewal of the medium every 2 days until the cells became confluent. Then the cells were harvested, seeded at a density of 10^5/dish in 2 ml of the medium in Corning dishes (35 × 10 mm) and incubated for about 8 days until they reached confluency. The medium was renewed every 2 days.

Treatment and incubation

Fatty acids were dissolved in absolute ethanol and diluted 1:1333 with the medium to give 90 μM solutions in 2 ml of medium. Radioactive fatty acids were diluted with unlabeled fatty acids to make 90 μM solutions in 2 ml of medium containing 1 μCi of fatty acids. Confluent smooth muscle cells were incubated in a CO_2 incubator in 2 ml of medium containing 90 μM labeled or unlabeled fatty acids for 16 hr. The same amount of ethanol added with fatty acids was introduced into control cultures. The

final ethanol concentration was 0.075%. After incubation, the dishes were placed on ice, the medium was aspirated, and the dishes were washed twice with ice-cold phosphate-buffered saline (pH 7.4), once with 2 ml of ice-cold solution containing 0.15 M NaCl, 50 mM Tris-chloride (pH 7.4), and 2 mg/ml of bovine albumin, and then twice with ice-cold phosphate-buffered saline (pH 7.4). Cells were collected with a rubber policeman in 2 ml of ice-cold phosphate-buffered saline (pH 7.4). An aliquot of the solution was used for protein assay, and the remainder was used for lipid analysis. Lipids in the solution were extracted with 8 ml of chloroform–methanol 2:1 (by vol) (27).

Lipid contents

Before extraction of the cell suspension, cholesteryl acetate was added as an internal standard. Cell lipids were extracted and analyzed using thin-layer chromatography and a flame ionization detector (28) using an Iatroscan TH-10 TLC/flame ionization analyzer (Diatron, Tokyo). Lipids were separated with the solvent system, hexane–ether–formic acid 54:5:0.05 (v/v) for neutral lipids and chloroform–methanol–water 60:20:2 for phospholipid subfractions. Values are expressed as μg/mg of cell protein.

Incorporation of radioactive fatty acids into lipids

Lipid fractions were separated by thin-layer chromatography on silica gel G (Merck, Darmstadt) by the method of Skipski et al. (29) with the following solvent systems: first step, isopropylether–acetic acid 96:4 (v/v); second step, petroleum ether–ether–acetic acid 90:10:1 (v/v) for neutral lipids (System I), and chloroform–methanol–water–acetic acid 25:15:4:2 (v/v) for phospholipid subfractions (System II). Lipids at the origin in system I were considered as total phospholipids. The sum of the radioactivity in phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), sphingomyelin, and lysophosphatidylcholine separated with system II was about 95% of that of the total phospholipids. Because PI and PS were not completely separable in system II, the sum of these fractions is shown as PI plus PS. The plates were stained with I_2 and each fraction was scraped into a scintillation vial and its radioactivity was measured.

Fatty acid composition

Lipids were separated by TLC as described above. Total phospholipids were obtained with system I, and phospholipid subfractions were obtained with system II. Each fraction was scraped into a tube and extracted with 8 ml of chloroform–methanol 2:1 (v/v). The solvents were then evaporated under N_2. Hydrolysis of phospholipids and methylation of the fatty acids were achieved by incubating the extracts with 1 ml of 0.5 N NaOH in 100% methanol for 30 min at room temperature. After incubation, the mixture was neutralized with 6 N HCl and the methylated fatty acids were extracted with 2 ml of hexane. The fatty acid composition was determined by gas–liquid chromatography in a GC7-A model apparatus (Shimadzu Co., Kyoto) equipped with a 2 m × 2 mm i.d. glass column packed with 15% DEGS, and a flame ionization detector. The column temperature was 190°C. Nitrogen was used as carrier gas and the flow rate was 50 ml/min. Peaks were identified by comparison with those of standards (Nu-Chek-Prep Co.). Peak areas were calculated electronically with a computing integrator (Chromatopac CRIA; Shimadzu Co.). In some experiments AA and 20:3 (n–3) were further analyzed by capillary gas–liquid chromatography (REG-HT Bonded Column, 0.35 mm i.d. × 30 m (Gasukuro Kogyo, Inc., Tokyo); carrier gas, helium, 0.8 kg/cm^2, 2.38 ml/min; column temperature, 180°C to 230°C, increased by 1°C/min).

Incorporation of radioactive fatty acids into cells

Confluent cells were incubated with radioactive fatty acids for 1 to 24 hr and the dishes were washed five times as described above. Cells were collected with a rubber policeman and radioactivity was measured.

Protein

Protein was determined by the method of Lowry et al. (30).

Statistics

The significance of differences of mean values was evaluated by Student's t test.

RESULTS

Lipid contents of smooth muscle cells

Cultured rabbit smooth muscle cells treated with 0.075% ethanol (control) contained a large amount of phospholipid and small amounts of free cholesterol and triglyceride (**Table 1**). After treatment with 90 μM concentrations of n–3 fatty acids for 16 hr, the protein content of the cells did not change significantly. The amounts of phospholipid and free cholesterol per mg of cellular protein in the cells did not change after treatment with LLA or EPA, but the amounts in cells treated with DHA were slightly less than those in control cells. The amount of triglyceride significantly increased in each group, the content increasing with the increase in unsaturation and chain length. The amounts of phospholipid subfractions did not significantly change after treatment with any fatty acid (**Table 2**).

TABLE 1. Effects of fatty acid treatment on the concentrations of proteins and lipids in smooth muscle cells[a]

	Control (n = 20)	18:3 (n-3) (n = 20)	20:5 (n-3) (n = 13)	22:6 (n-3) (n = 9)
Protein (μg/dish)	431 ± 14	428 ± 16	441 ± 8	442 ± 20
Lipids (μg/mg cell protein)				
Phospholipid	148 ± 6	143 ± 8	150 ± 9	125 ± 2[b]
Free cholesterol	14.5 ± 0.5	13.3 ± 0.7	13.7 ± 0.7	11.0 ± 0.4[b]
Triglyceride	5.7 ± 0.7	11.3 ± 1.2[c]	19.6 ± 2.5[c]	22.3 ± 2.3[c]

[a]Confluent smooth muscle cells were incubated with 90 μM free fatty acids or ethanol alone (control) for 16 hr. Values are means ± SE.
[b]$P < 0.005$.
[c]$P < 0.001$.

Incorporation of radioactive fatty acids into smooth muscle cells

Next, 90 μM radioactive fatty acids were incubated with smooth muscle cells for different periods and the incorporation of fatty acid was calculated from the specific activities of fatty acids (**Fig. 1**). All polyunsaturated fatty acids tested were incorporated into cells in a time-dependent fashion for 16 hr, but the rates of incorporation after 16 hr were much smaller than before 16 hr. With the exception of LLA, more than 50% of fatty acids added to the medium were used up within 16 hr. The incorporations varied slightly depending on the primary cultures; standard deviations of the incorporation were 2–16% of the means for all fatty acids. In subsequent experiments, therefore, a 16-hr incubation period was used. The amounts of incorporation of the five fatty acids tested differed: AA was incorporated to the greatest extent, followed by DHA, LA, EPA (slightly less), and LLA, the least.

Incorporation of radioactive fatty acids into lipids in smooth muscle cells

The total incorporation of fatty acids into lipids was highest with AA (260 nmol/mg of cell protein) and lowest with LLA (73 nmol/mg of cell protein) (**Table 3**). The distribution of radioactivity in lipid subfractions was almost the same with all the fatty acids: 62.4–70.1% of the fatty acids were incorporated into triglyceride and 27.4–29.7% into phospholipids. Incorporation into other fractions was low.

Incorporation of radioactive fatty acids into phospholipid subfractions in smooth muscle cells

Table 4 shows the relative incorporation of various polyunsaturated fatty acids into phospholipid subfractions. The patterns of incorporation of these fatty acids differed. The incorporation of all five fatty acids was greatest into PC (60.9–79.4% of the total incorporation into phospholipids). Compared with LA and LLA, however, AA, EPA, and DHA were incorporated into PC to a lesser extent and there was a greater incorporation of AA and EPA into PE and PI + PS, and of DHA into PE. DHA was not incorporated into PI + PS as well as AA and EPA were. The incorporation patterns of LLA and LA and of EPA and AA were only slightly different; the n-3 fatty acids were incorporated into PE more and into PI + PS less than their n-6 counterparts. The incorporation of all fatty acids into sphingomyelin and lysoPC was very low.

Modification of fatty acid composition of phospholipids by n-3 fatty acids

The phospholipids of the control smooth muscle cells contained mainly palmitic and stearic acids (saturated),

TABLE 2. Effects of fatty acid treatment on the concentrations of phospholipid subfractions in smooth muscle cells[a]

	Control (n = 4)	18:3 (n-3) (n = 4)	20:5 (n-3) (n = 4)	22:6 (n-3) (n = 4)
Phosphatidylcholine	95.2 ± 1.0[b]	93.0 ± 3.2	99.1 ± 7.5	91.8 ± 8.7
Phosphatidylinositol + phosphatidylserine	7.2 ± 0.4	7.2 ± 0.7	5.3 ± 0.9	5.3 ± 0.9
Phosphatidylethanolamine	30.8 ± 2.3	30.6 ± 0.8	30.5 ± 1.8	27.5 ± 1.9

[a]Confluent smooth muscle cells were incubated with 90 μM free fatty acids or ethanol alone (control) for 16 hr.
[b]Values are mean ± SE (μg/mg cell protein). These data were obtained with a different primary culture from that for Table 1.

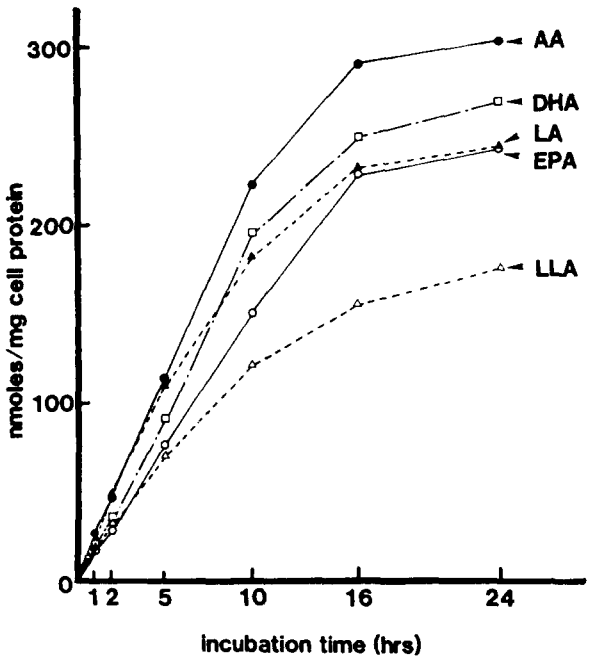

Fig. 1. Radiolabeled fatty acids (90μM) (5.56 mCi/mmol) were incubated with confluent smooth muscle cells for the indicated periods. The radioactivity in the cell was measured and the incorporation of fatty acids into cells was calculated from the specific activity. Data are averages for values in two separate cultures. Two values at each period did not differ more than 10% from each other.

oleic acid (monoene), and AA (polyene) (**Table 5,** control). Small amounts of other polyunsaturated fatty acids such as LA, docosapentaenoic acid (n-3), and DHA were also found, but no LLA or EPA was detectable. After treatment with 90 μM LLA, the LLA content increased to 12.1%, while the palmitic and oleic acid contents were significantly decreased, and the apparent AA content was not affected. Since 20:3 (n-3), an elongation metabolite of LLA, exhibits a gas-liquid chromatographic retention time close to that of AA, these two fatty acids were further separated in some samples by capillary gas-liquid chromatography. AA content was 16.7 ± 0.3(%) (mean ± SE) and 20:3 (n-3) was not detected in the control. AA and 20:3 (n-3) contents on treatment with LLA were 15.9 ± 0.5(%) and 2.0 ± 0.2(%), respectively. AA content did not significantly decrease on treatment with LLA in spite of a small increase in 20:3 (n-3) content. Desaturated metabolites of LLA, such as 20:5(n-3) (EPA), 22:5(n-3), and 22:6(n-3) (DHA) were not increased appreciably, although the EPA content did increase from 0 to 0.3%. After treatment with LLA, polyene fatty acids increased at the expense of saturated and monoenoic acids, while the contents of n-6 fatty acids were not affected (Table 5).

After treatment with 90 μM EPA, the contents of EPA and its elongated metabolite, docosapentaenoic acid, increased significantly. The main fatty acid that decreased was AA. With this treatment, increase in polyene fatty acids was only moderate, because n-6 fatty acids decreased in spite of an increase in n-3 fatty acids.

After treatment with DHA, DHA content increased markedly. AA and oleic acid were the main fatty acids that decreased. With this treatment, polyene fatty acids increased markedly at the expense of n-6 fatty acids and monoenes.

Fatty acid modification of phospholipid subfractions by n-3 fatty acids

The major fatty acids in PC were palmitic and oleic acids (**Table 6,** control). The AA content was low

TABLE 3. Incorporation of radioactive fatty acids into lipids in smooth muscle cells[a]

	18:2(n-6) (n = 3)	18:3(n-3) (n = 3)	20:4(n-6) (n = 3)	20:5(n-3) (n = 3)	22:6(n-3) (n = 3)
			nmoles/mg cell protein		
Phospholipid	48.6 ± 2.0 (27.4[b])	21.7 ± 1.7 (29.7)	75.9 ± 2.4 (29.2)	61.0 ± 0.1 (28.1)	67.4 ± 0.8 (27.5)
Monoglyceride	0.2 ± 0.02 (0.1)	0.1 ± 0.02 (0.1)	0.6 ± 0.03 (0.2)	0.4 ± 0.1 (0.2)	0.7 ± 0.04 (0.2)
Diglyceride	1.3 ± 0.2 (0.8)	0.5 ± 0.04 (0.7)	4.0 ± 0.1 (1.5)	2.6 ± 0.02 (1.2)	3.1 ± 0.1 (1.3)
Free fatty acid	2.2 ± 0.3 (1.3)	1.0 ± 0.04 (1.3)	7.3 ± 1.1 (2.8)	8.2 ± 1.3 (3.8)	18.1 ± 2.2 (7.4)
Triglyceride	124.0 ± 7.0 (70.1)	49.3 ± 3.6 (67.5)	167.0 ± 1.2 (64.3)	142.0 ± 2.8 (65.4)	153.0 ± 7.1 (62.4)
Cholesteryl ester	0.8 ± 0.4 (0.5)	0.5 ± 0.1 (0.6)	4.8 ± 0.03 (1.8)	3.3 ± 0.4 (1.5)	3.1 ± 0.04 (1.3)
Total	178	73	260	217	245

[a]Confluent cells were incubated with 90 μM [1-^{14}C]-labeled fatty acids (5.56 mCi/mmol) for 16 hr. Values are means ± SE.
[b]Values in parentheses indicate percentages of the total incorporation into lipids.

TABLE 4. Incorporation of radioactive fatty acids into phospholipid subfractions in smooth muscle cells[a]

	18:2(n-6) (n = 3)	18:3(n-3) (n = 3)	20:4(n-6) (n = 3)	20:5(n-3) (n = 3)	22:6(n-3) (n = 3)
			%		
Phosphatidylcholine	78.4 ± 0.5[b]	79.4 ± 0.9	61.8 ± 0.2	60.9 ± 0.3	65.6 ± 0.3
Phosphatidylinositol + phosphatidylserine	10.7 ± 0.5	8.2 ± 0.2	18.1 ± 0.3	16.3 ± 0.6	9.6 ± 0
Phosphatidylethanolamine	8.9 ± 0.1	10.1 ± 0.9	18.6 ± 0.2	21.1 ± 0.3	21.9 ± 0.3
Sphingomyelin	1.5 ± 0.03	1.3 ± 0.1	0.8 ± 0.07	1.2 ± 0.1	1.4 ± 0.1
Lysophosphatidylcholine	0.4 ± 0.03	0.5 ± 0.05	0.7 ± 0.06	0.7 ± 0.03	1.5 ± 0.1

[a]Values were obtained in the same experiment as those in Table 3 and are shown as means ± SE.
[b]Values are percentages of the total phospholipids, calculated as the sum of the subfractions in Table 4. The sum of the amounts in the phospholipid subfractions was more than 95% of the total amount in phospholipid shown in Table 3.

compared with that in PE or PI + PS (Tables 6-8, control). In PI + PS, the major fatty acids were stearic, oleic, and arachidonic (**Table 7,** control); in PE, the major acids were oleic and arachidonic acids (**Table 8,** control). DHA and docosapentaenoic acid were mainly found in PE, and linoleic acid was highest in PC.

After treatment with LLA, the largest increase in this acid was in PC and the smallest was in PI + PS; the apparent AA content of all subfractions was not significantly affected. The palmitic acid content of PC decreased, and the oleic acid contents of PC and PE decreased slightly, but not significantly.

After treatment with EPA, EPA content and that of docosapentaenoic acid were increased most in PE and less in PC and PI + PS. The AA content of PE and PI + PS was markedly decreased. The decrease in PC was small and not statistically significant, but the palmitic and oleic acid contents of PE and PI + PS, respectively, were significantly decreased.

After treatment with DHA, DHA content increased most in PE and less in PC and PI + PS. AA decreased markedly in PE and moderately in PI + PS and did not change in PC. Oleic acid was decreased significantly in PE and slightly but not significantly in PC.

Modification of fatty acid composition of phospholipids by n-6 fatty acids

To clarify the elongation or desaturation activities of the smooth muscle cells, the modification of fatty acid composition of phospholipids after treatment with LA or

TABLE 5. Effects of (n-3) fatty acids on the fatty acid composition of phospholipids in smooth muscle cells[a]

	Control (n = 4)	18:3 (n-3) (n = 4)	20:5 (n-3) (n = 4)	22:6 (n-3) (n = 4)
		%		
16:0	16.5 ± 0.4	12.9 ± 0.1[b]	13.5 ± 0.3[b]	13.7 ± 1.1
16:1 (n-7)	2.7 ± 0.2	1.4 ± 0.3[b]	1.5 ± 0.4[b]	1.7 ± 0.3[b]
18:0	15.2 ± 0.5	15.8 ± 0.1	16.1 ± 0.3	12.9 ± 0.7[b]
18:1 (n-9)	25.5 ± 0.5	21.0 ± 0.4[b]	24.2 ± 0.3	18.9 ± 0.5[b]
18:2 (n-6)	4.3 ± 0.1	3.8 ± 0.1[b]	4.6 ± 0.5	4.0 ± 0.2
18:3 (n-3)	n.d.[c]	12.1 ± 0.6[b]	n.d.	n.d.
20:3 (n-6)	1.3 ± 0.1	1.0 ± 0[b]	1.0 ± 0.1[b]	0.8 ± 0.1[b]
20:4 (n-6)	16.9 ± 0.3	17.8 ± 0.3[d]	11.7 ± 0.3[b]	10.7 ± 0.1[b]
20:4 (n-3)	n.d.	n.d.	n.d.	n.d.
20:5 (n-3)	n.d.	0.3 ± 0.1[b]	6.6 ± 0.2[b]	0.5 ± 0.1[b]
22:4 (n-6)	1.9 ± 0.1	1.4 ± 0.1[b]	1.3 ± 0.1[b]	1.0 ± 0.1[b]
22:5 (n-3)	6.0 ± 0.2	5.0 ± 0.1[b]	10.7 ± 0.2[b]	3.9 ± 0.1[b]
22:6 (n-3)	6.4 ± 0.2	5.3 ± 0.2[b]	5.5 ± 0.5	28.6 ± 1.5[b]
Saturate	31.7	28.7[b]	29.6	26.6[b]
Monoene	28.2	22.4[b]	25.7[b]	20.6[b]
Polyene	36.8	46.7[b]	41.4[b]	49.5[b]
n-6	23.1	23.0	17.6[b]	15.7[b]
n-3	12.4	22.7[b]	22.8[b]	33.0[b]

[a]Confluent smooth muscle cells were incubated with 90 μM fatty acids or ethanol alone for 16 hr. Values are means ± SE. Minor components are not listed in the Table.
[b]Significantly different from control.
[c]Not detectable.
[d]Small amounts of 20:3 (n-3) are included (see the text).

TABLE 6. Effects of (n-3) fatty acids on the fatty acid composition of phosphatidylcholine in smooth muscle cells[a]

	Control (n = 3[b])	18:3 (n-3) (n = 3)	20:5 (n-3) (n = 3)	22:6 (n-3) (n = 3)
			%	
16:0	25.6 ± 0.6	19.3 ± 0.5[c]	24.7 ± 1.6	27.3 ± 0.3
16:1 (n-7)	8.2 ± 0.2	5.2 ± 0.2	6.9 ± 0.8	8.3 ± 0.8
18:0	6.3 ± 0.9	6.0 ± 0.6	6.4 ± 0.3	7.7 ± 0.6
18:1 (n-9)	37.4 ± 1.9	33.4 ± 1.8	37.1 ± 1.0	34.4 ± 2.3
18:2 (n-6)	5.7 ± 2.2	3.5 ± 0.2	5.0 ± 1.3	3.1 ± 0.7
18:3 (n-3)	n.d.[d]	22.9 ± 1.7[c]	n.d.	n.d.
20:3 (n-6)	0.5 ± 0.2	0.4 ± 0.1	0.5 ± 0.1	0.8 ± 0.2
20:4 (n-6)	4.3 ± 1.0	3.9 ± 0.5[e]	3.3 ± 0.2	4.8 ± 0.5
20:4 (n-3)	n.d.	n.d.	n.d.	n.d.
20:5 (n-3)	n.d.	n.d.	5.0 ± 0.3[c]	n.d.
22:4 (n-6)	0.7 ± 0.2	0.2 ± 0.1	0.4 ± 0.2	0.3 ± 0.2
22:5 (n-3)	0.6 ± 0.1	0.4 ± 0.2	5.6 ± 0.6[c]	1.3 ± 0.2
22:6 (n-3)	1.5 ± 0.2	0.2 ± 0.2[e]	1.4 ± 0.3	7.2 ± 0.8[c]

[a]Confluent cells were incubated with 90 μM fatty acids or ethanol alone (control) for 16 hr. Values are means ± SE. These data were obtained with a different primary culture from that for Table 4.
[b]Nine separate cultures were combined to make three samples.
[c]Significantly different from control.
[d]Not detectable.
[e]Small amount of 20:3 (n-3) might be included.

AA was examined (**Table 9**). After treatment with LA, LA content increased markedly and its elongation metabolite, 20:2 (n-6), increased slightly. Desaturation metabolites such as 20:3 (n-6) and AA did not change significantly. After treatment with AA, AA content slightly increased and its elongation metabolite, adrenic acid, increased significantly.

DISCUSSION

Table 4 shows clearly that the fatty acid chain length is more important than the fatty acid family itself in the metabolism in phospholipids, inasmuch as the patterns of incorporation of LLA and EPA into phospholipid classes were similar to those of LA and AA, respectively. Previously we found that EPA and AA were metabolized in a similar manner in rat aorta (31). LLA and LA were incorporated to a greater extent than EPA and AA into PC, whereas the reverse pattern of incorporation into PE and PI + PS was noted. DHA differed in that it was incorporated into PE more than LLA and LA and into PI + PS less than EPA and AA. In spite of the differences mentioned above, all of the radioactive fatty acids were incorporated most into PC. This does not mean that these fatty acids all have the highest affinity to PC, because the

TABLE 7. Effects of (n-3) fatty acids on the fatty acid composition of (phosphatidylinositol + phosphatidylserine) in smooth muscle cells[a]

	Control (n = 3[b])	18:3 (n-3) (n = 3)	20:5 (n-3) (n = 3)	22:6 (n-3) (n = 3)
			%	
16:0	5.1 ± 0.6	3.7 ± 0.8	4.0 ± 0.2	4.6 ± 0.3
16:1 (n-7)	1.9 ± 0.2	1.1 ± 0.2	1.3 ± 0.5	1.2 ± 0.1
18:0	26.9 ± 2.3	30.7 ± 1.1	25.0 ± 0.4	25.1 ± 0.8
18:1 (n-9)	26.3 ± 1.5	25.2 ± 6.4	20.7 ± 0.6[c]	25.5 ± 1.0
18:2 (n-6)	1.4 ± 0.5	1.7 ± 0.3	0.7 ± 0.2	1.0 ± 0.1
18:3 (n-3)	n.d.[d]	6.4 ± 0.8[c]	n.d.	n.d.
20:3 (n-6)	0.9 ± 0.2	0.8 ± 0.2	0.3 ± 0.2	0.5 ± 0
20:4 (n-6)	21.3 ± 0.8	22.5 ± 2.1[e]	15.0 ± 0.3[c]	16.2 ± 1.3[c]
20:4 (n-3)	n.d.	n.d.	n.d.	n.d.
20:5 (n-3)	n.d.	n.d.	6.3 ± 0.4[c]	n.d.
22:4 (n-6)	n.d.	0.9 ± 0.2	0.2 ± 0.1	1.5 ± 0.2
22:5 (n-3)	1.9 ± 0.3	2.4 ± 0.5	6.5 ± 1.0[c]	1.8 ± 0
22:6 (n-3)	n.d.	n.d.	0.4 ± 0.2	6.9 ± 0.2[c]

[a,b,c,d,e]See legend to Table 6 for explanation.

TABLE 8. Effects of (n-3) fatty acids on the fatty acid composition of phosphatidylethanolamine in smooth muscle cells[a]

	Control (n = 3[b])	18:3 (n-3) (n = 3)	20:5 (n-3) (n = 3)	22:6 (n-3) (n = 3)
			%	
16:0	8.3 ± 1.4	5.4 ± 0.5	3.8 ± 0.1[c]	6.1 ± 0.6
16:1 (n-7)	2.3 ± 0.1	1.8 ± 0.3	1.9 ± 0	2.5 ± 0.5
18:0	14.5 ± 0.1	15.6 ± 0.8	13.8 ± 0.2	14.3 ± 1.0
18:1 (n-9)	31.9 ± 2.1	26.7 ± 2.1	27.4 ± 0.3[c]	26.3 ± 1.9[c]
18:2 (n-6)	2.7 ± 0.3	2.9 ± 0.5	1.7 ± 0.8	5.3 ± 1.9
18:3 (n-3)	n.d.[d]	12.0 ± 0.3[c]	n.d.	n.d.
20:3 (n-6)	0.7 ± 0.1	0.8 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
20:4 (n-6)	20.7 ± 1.9	16.9 ± 0.9[c]	13.3 ± 0.3[c]	11.5 ± 0.2[c]
20:4 (n-3)	n.d.	n.d.	n.d.	n.d.
20:5 (n-3)	n.d.	n.d.	9.1 ± 0[c]	n.d.
22:4 (n-6)	2.2 ± 0.2	1.9 ± 0.2	2.1 ± 0.1	1.9 ± 0.4
22:5 (n-3)	5.1 ± 1.0	6.5 ± 0.6	14.0 ± 0.7[c]	5.1 ± 0.5
22:6 (n-3)	5.2 ± 0.4	6.2 ± 1.1	8.9 ± 0.8	19.9 ± 0.6[c]

[a,b,c,d,e] See legend to Table 6 for explanation.

actual contents of these fatty acids were not always highest in PC (Tables 6-8). Similar discrepancies were observed in fibroblasts (32) and platelets (23). The explanation of these findings is not clear, but could be due to differences in the masses and turnover rates of the individual phospholipid classes. Relative specific activities calculated from the data in Tables 2 and 4 (% of radioactivity in a lipid/μg of lipid) are shown in **Table 10.** Specific activities for all three fatty acids were highest in PI +PS. Specific activity of 18:3(n-3) in PC was higher than in PE, but activities of 20:5(n-3)[+22:5(n-3)] and DHA in PE were slightly higher than in PC. These data partly, but not completely, explain the above discrepancies. High specific activities in PI + PS suggest that different turnover (deacylation) rates for individual phospholipids were also reflected in the data in Tables 4 and 6-8.

The incorporation patterns of the various unlabeled fatty acids shown in Tables 6-8 confirm the specificity of the fatty acid affinity to phospholipid subfractions suggested from Table 4: LLA and LA to PC, AA to PE and PI + PS, and EPA and DHA to PE in cultured rabbit smooth muscle cells (Tables 6-8). Data in other tissues and cell lines are fairly consistent with these specificities in smooth muscle cells (16, 22, 23, 33-38). However, there seem to be some differences in terms of regulation of fatty acid acylation and deacylation in phospholipids between smooth muscle cells and endothelial cells which compose the vessel wall. One paper on human umbilical vein endothelial cells reported that PC was most enriched with AA or EPA among phospholipid subfractions and EPA replaced AA in PC to a remarkable degree. Although the phospholipid pool of prostaglandin precursor fatty acids is not completely understood in these cell lines, these differences may cause different prostaglandin metabolism such as rapid synthesis of PGI_2 in endothelial cells (17) and rather slow and continuous synthesis in smooth muscle cells (39).

The specificity of the fatty acid affinity to phospholipid classes in rabbit smooth muscle cells may partly explain why LLA and LA did not replace AA in phospholipids, but EPA and DHA did (Tables 5 and 9). PC, into which LLA and LA are preferentially incorporated, is not rich in AA (Table 6). On the other hand, PE, into which EPA and DHA are incorporated to the greatest degree, is also rich in AA. DHA and EPA markedly decreased the AA content of PE, with a smaller effect on PI + PS, and a negligible effect on PC. DHA had the same ability as EPA to reduce the AA content of phospholipids (Table 5), but unlike EPA, it reduced the content of oleic acid as well as of AA.

In general, cultured cells have elongation activity but decreased desaturase activity, with the extent of decrease

TABLE 9. Effects of 18:2 (n-6) and 20:4 (n-6) on the fatty acid composition of phospholipids[a]

	18:2 (n-6)		20:4 (n-6)	
	Control (n = 5)	Treatment (n = 5)	Control (n = 4)	Treatment (n = 5)
18:2 (n-6)[b]	5.4	26.9[c]	5.1	4.3
18:3 (n-6)	2.4	2.7	n.d.	n.d.
20:2 (n-6)	n.d.	1.1[c]	n.d.	n.d.
20:3 (n-6)	n.d.	n.d.	1.5	1.0
20:4 (n-6)	12.9	11.8	16.3	19.7[c]
22.4 (n-6)	1.7	1.7	1.1	3.1[c]

[a] Confluent smooth muscle cells were incubated with 90 μM free fatty acids or ethanol (control) for 16 hr. Different primary cultures were used for 18:2 (n-6) and 20:4 (n-6) experiments. Values are means for four to five samples. Standard deviations were less than 10% of the mean.
[b] Only n-6 family fatty acids are listed.
[c] Significantly increased.

TABLE 10. Relative specific activities for (n−3) fatty acids in phospholipid subfractions

Phospholipid	Treatment		
	18:3 (n-3)	20:5 (n-3)[a]	22:6 (n-3)
PC	2.03[b]	1.43	1.66
PI + PS	2.73	7.09	4.36
PE	0.78	1.61	1.85

[a] The values in this group show relative specific activities for both 20:5 (n-3) and 22:5 (n-3) because some 20:5 (n-3) was elongated to 22:5 (n-3).

[b] $\frac{\text{Percent of radioactivities in a lipid (\%)}}{\text{Amount of lipid } (\mu g/\text{dish})}$ These data were obtained from Table 2 and Table 4.

depending on the cell line (40). The results shown in Tables 5-9 suggest that cultured rabbit aortic smooth muscle cells have strong elongation activity and very weak Δ6 and Δ4 desaturase activity. The analysis of fatty acid composition of total lipids confirmed this conclusion (data not shown). Cultured guinea pig aortic smooth muscle cells do not have Δ6, Δ5 or Δ4 desaturase activity (15, 41).

The above results suggest that LLA administration will not reduce the AA content of phospholipids in the aorta unless it is metabolized to EPA or DHA in tissues other than aorta. Dyerberg et al. (19) reported that administration of linseed oil for 7 days did not increase EPA or DHA in human plasma. In other animal and human experiments, however, LLA increased the contents of EPA and DHA (20-23). Thus the period of fatty acid administration may affect the results. Moreover, although the present study and other reports show that cultured aortic cells, such as smooth muscle cells (15, 41) and endothelial cells (42), have weak Δ6 desaturase activity, the possibility that the aorta can convert LLA to EPA or DHA in vivo, especially over a long period, cannot be completely excluded. When EPA and DHA are formed from LLA, however, LLA can reduce the AA content of phospholipids, probably because EPA and DHA synthesized from LLA replaces AA (20-23).

Modifications of fatty acids of phospholipids in smooth muscle cells may affect prostaglandin metabolism and membrane functions. Subsequent papers will report on these biological phenomena.

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