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Cellular interactions between n-6 and n-3 fatty acids: a mass analysis of fatty acid elongation/desaturation, distribution among complex lipids, and conversion to eicosanoids

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Abstract The biologic effect of eicosanoids depends in large measure upon the relative masses in tissues of eicosanoids derived from the n-6 fatty acids, dihomogammalinolenic acid and arachidonic acid, and the n-3 fatty acid, eicosapentaenoic acid. Generation of this tissue balance is related to the relative cellular masses of these precursor fatty acids, the competition between them for entry into and release from cellular phospholipids, and their competition for the enzymes that catalyze their conversion to eicosanoids. In order to better understand these processes, we studied the cellular interactions of n-6 and n-3 fatty acids using an essential fatty acid-deficient, PGE-producing, mouse fibrosarcoma cell line, EFD-1. Unlike studies using cells with endogenous pools of n-6 and n-3 fatty acids, the use of EFD-1 cells enabled us to examine the metabolic fate of each family of fatty acids both in the presence and in the absence of the second family of fatty acids. Thus, the specific effects of one fatty acid family on the other could be directly assessed. In addition, we were able to replete the cells with dihomogammalinolenic acid (DHLA), arachidonic acid (AA), and eicosapentaenoic acid (EPA) of known specific activities; thus the masses of cellular DHLA, AA, and EPA, and their metabolites, PGE_1, PGE_2, and PGE_3, respectively, could be accurately quantitated. The major findings of this study were: *1)* n-6 fatty acids markedly stimulated the elongation of EPA to 22:5 whereas n-3 fatty acids inhibited the Δ5 desaturation of DHLA to AA and the elongation of AA to 22:4; *2)* n-6 fatty acids caused a specific redistribution of cellular EPA from phospholipid to triacylglycerol; *3)* n-3 fatty acids reduced the mass of DHLA and AA only in phosphatidylinositol whereas n-6 fatty acids reduced the mass of EPA to a similar extent in all cellular phospholipids; and *4)* n-3 fatty acids caused an identical (33%) reduction in the bradykinin-induced release of PGE_1 and PGE_2, whereas n-6 fatty acids stimulated PGE_3 release 2.3-fold. Together, these highly quantitative metabolic data increase our understanding of the regulation of both the cellular levels of DHLA, AA, and EPA, and their availability for eicosanoid synthesis. In addition, these findings provide a context for the effective use of these fatty acids in dietary therapies directed at modulation of eicosanoid production.—**Rubin, D., and M. Laposata.** Cellular interactions between n-6 and n-3 fatty acids: a mass analysis of fatty acid elongation/desaturation, distribution among complex lipids, and conversion to eicosanoids. *J. Lipid Res.* 1992. **33:** 1431–1440.

Supplementary key words prostaglandin • phospholipid • dihomogammalinolenic acid • arachidonic acid • eicosapentaenoic acid • prostaglandin E_1 • prostaglandin E_2 • prostaglandin E_3

In response to a variety of stimuli, the n-6 fatty acids, dihomogammalinolenic acid (DHLA, 20:3, Δ8, 11, 14) and arachidonic acid (AA, 20:4, Δ5, 8, 11, 14), and the n-3 fatty acid, eicosapentaenoic acid (EPA, 20:5, Δ5, 8, 11, 14, 17), are released from cellular phospholipids and converted to eicosanoids that differ both in the number of their double bonds (for prostaglandins, one, two, and three double bonds, respectively) and, in many instances, in their biologic actions (1–3). These eicosanoids critically influence a wide range of physiologic and pathologic processes, including inflammation and immunity (4–8), hemostasis (9, 10), blood pressure (10, 11), and atherosclerosis (12, 13). In addition, it is believed that the net biologic effect of eicosanoids is determined in large measure by the balance in tissues between eicosanoids derived from DHLA, AA, and EPA. Further, it is thought that the generation of this balance is regulated by complex interactions between DHLA, AA, and EPA at multiple levels of the prostaglandin biosynthetic pathway (1–3).

Abbreviations: DHLA, dihomogammalinolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PG, prostaglandin; PL, phospholipid; TG, triacylglycerol; CoA, coenzyme A; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; EP, ethanolamine plasmalogen; CP, choline plasmalogen; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; DPM, disintegrations per minute; AA/DHLA, desaturation of DHLA to AA; 22:4/AA, elongation of AA to 22:4; 22:5/EPA, elongation of EPA to 22:5.

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The metabolic interplay of n-6 and n-3 fatty acids has been the focus of much recent research, but significant gaps in our knowledge remain. Numerous in vitro and in vivo studies have shown that n-3 fatty acids reduce both the level of AA in cellular phospholipids (14-17) and the production of AA-derived eicosanoids (14, 17-19). Analysis of the effects of n-3 fatty acids on AA metabolism is technically straightforward because AA is the predominant eicosanoid precursor fatty acid in vivo, and in most cell types, n-3 fatty acids are either undetectable or present in extremely small amounts (3). Hence, analysis of the effect of n-3 fatty acids on AA metabolism can be achieved simply by supplementing cells with n-3 fatty acids. In contrast, the presence of significant endogenous stores of AA in most cell types has greatly limited the study of the specific effects of AA on EPA metabolism. Similarly, little is known concerning the metabolic interactions of DHLA and EPA, including the effect of EPA on one-series prostaglandin production. These questions are of particular importance in light of the documented salutary effect of dietary EPA on a variety of cardiovascular processes (10-13), and of PGE_1 (4, 5), DHLA (20, 21), and EPA (6-8) on a range of inflammatory disorders. An additional factor that has limited our understanding of the metabolic interactions of AA, DHLA, and EPA has been a lack of mass data concerning both cellular fatty acid metabolism and eicosanoid production. Many studies in this area have used radioactive fatty acid substrates, which, when incorporated into cells, mix with endogenous eicosanoid precursor fatty acids. Thus, the specific activity of incorporated fatty acids and their eicosanoid metabolites is unknown, and measured radioactivity cannot be reliably converted to mass. In addition, it has become clear that the phospholipid distribution of incorporated radiolabeled fatty acids may differ markedly from that of endogenous fatty acids (22). Studies that have attempted to avoid the problems related to the use of isotopic fatty acids by using unlabeled fatty acid substrates have been hampered by the difficulty in accurately measuring the extremely small masses of their prostaglandin metabolites which are generated in cell culture systems (23, 24).

With these issues in mind, we performed a mass analysis of the reciprocal metabolic interactions of n-6 and n-3 fatty acids in a unique, essential fatty acid-deficient, PGE-producing cell line, EFD-1. This cell line, which is a derivative of the mouse fibrosarcoma cell line, $HSDM_1C_1$ (25), provided us with several important advantages. First, the absence of endogenous n-6 or n-3 fatty acids allowed us to examine the metabolic fate of each family of fatty acids both in the absence and in the presence of the second family of fatty acids. Thus, the specific effects of one fatty acid family on the other could be directly assessed. Second, because there is no endogenous pool of eicosanoid precursor fatty acids in these cells, the specific activity of incorporated radioactive precursor fatty acids and released prostaglandins is known. Hence, DPM can be converted directly to mass, and masses as low as 0.2 pmol can be accurately measured. Third, quantifying eicosanoid production is simplified using EFD-1 cells because they produce only a single eicosanoid, PGE ($>$ 95% of eicosanoid production). We report a series of highly specific cellular interactions between n-6 and n-3 fatty acids, involving fatty acid desaturation and elongation, partitioning between cellular phospholipids, triacylglycerol and cholesteryl ester, distribution among individual cellular phospholipids, and eicosanoid production. Together, these findings increase our understanding of the regulation of both the cellular levels of DHLA, AA, and EPA and their availability for eicosanoid synthesis, and, further, these results may assist in the development of rational dietary therapies using these polyunsaturated fatty acids.

MATERIALS AND METHODS

Cell culture

The EFD-1 cell line was maintained continuously in delipidated medium that consisted of serum-free Ham's F-10 medium (GIBCO) with 4.2% delipidated horse serum, 1% delipidated fetal calf serum, 10 mM HEPES buffer (Sigma), 170 units/ml penicillin, 170 μg/ml streptomycin, and 2 mM glutamine. Dialyzed horse and fetal calf sera were delipidated according to the method of Capriotti and Laposata (26). Sera, antibiotics, and glutamine were purchased from GIBCO.

Supplementation of EFD-1 cells with n-6 and n-3 fatty acids

Delipidated medium was supplemented with varying concentrations of DHLA (Nu-Chek) and/or EPA (Nu-Chek) and 2 mg/ml fatty acid-free bovine serum albumin (Sigma). The fatty acids were added to delipidated medium in a small volume of ethanol (final ethanol concentration $<$ 0.1% (v/v)). EFD-1 cells were grown to near confluence in 35, 60, or 100 mm diameter petri dishes (Corning) in delipidated medium, and then incubated for various times with fatty acid-supplemented medium. The incubation volumes were 1.25 ml, 3 ml, and 8 ml for 35, 60, and 100 mm diameter dishes, respectively. At the designated times, the cell monolayers were washed four times with ice-cold Dulbecco's phosphate-buffered saline (PBS), and the cells were scraped on ice into 1 ml of ice-cold PBS with a rubber policeman. This method of cell harvesting has been shown to preserve cellular AA (27). Cells from companion plates were counted with a hemocytometer.

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Lipid analysis

After fatty acid incubations, cellular lipids were extracted by the method of Cohen et al. (28) for analysis of the total cellular fatty acid content and for analysis of fatty acid distribution among cellular phospholipid, triacylglycerol, and cholesteryl ester. For analysis of the distribution of incorporated fatty acids among individual phospholipid species, cellular lipids were extracted in the presence of acid to convert plasmalogen species to lysophospholipids (29). Cellular phospholipid, triacylglycerol, and cholesteryl ester were separated on silica gel 60 thin-layer chromatography (TLC) plates (E. Merck) using a system of hexane–diethyl ether–acetic acid 70:30:1. Phospholipid species were separated on the same TLC plates in two dimensions: chloroform–methanol–ammonium hydroxide 65:25:5 followed by chloroform–methanol–ammonium hydroxide 65:35:5 in the first dimension, and chloroform–acetone–methanol–acetic acid–water 50:20:10:10:2.5 in the second dimension. In an atmosphere of nitrogen, the lipid moieties were visualized with iodine vapor and then scraped from the plate. In preliminary recovery experiments using gas chromatographic analysis, we and others in our lab (Hallaq, Y., and M. Laposata, unpublished observations) confirmed that this procedure did not result in loss of polyunsaturated fatty acids (data not shown). Fatty acid methyl esters were prepared for gas chromatography by the method of Turk et al. (30) either directly from the silica gel or from whole cell lipid extracts. Prior to lipid extraction, known quantities of tripentadecanoin (Nu-Chek), diheptadecanoyl phosphatidylcholine (Avanti), and cholesteryl linoleate (Nu-Chek), or myristate-containing phospholipid and lysophospholipid standards (Avanti) were added to the cell suspensions as internal standards. (EFD-1 cells contain only scant endogenous myristate (see Results).) Gas chromatography was performed on a Perkin-Elmer 8500 instrument with a WCOT capillary Supelcowax 10 column (Supelco). The temperature increased 10°C/min from 150°C to 250°C. Peaks were identified by comparison with retention times of known standards, and their areas were integrated.

Prostaglandin release studies

EFD-1 cells were grown to near confluence in 60-mm diameter petri dishes, and then incubated with either 20 μM [1-^{14}C]DHLA (47 μCi/μmol), 20 μM [1-^{14}C]EPA (40 μCi/μmol), or 20 μM of both labeled fatty acids (Du Pont–New England Nuclear) for 24 h. The cells were washed twice with pre-warmed PBS and then stimulated for 15 min at 37°C with bradykinin (10 μM) (Peninsula Labs) in 3 ml of PBS. The stimulation buffer was supplemented with 2 mg/ml fatty acid-free bovine serum albumin. Unlabeled PGE_1, PGE_2, and PGE_3 (Cayman Chemical) were added to the supernatants as carriers and unlabeled PGD_2 was added as an internal standard. Prostaglandins were extracted from the supernatants with ethyl acetate (31) after acidification with 1 M citric acid, and then separated on a 2.0 mm ID × 250 mm, Ultrasphere 5 mm C18 reverse phase HPLC column (Beckman). The solvent system consisted of 0.017 M H_3PO_4 and acetonitrile in a ratio of 70:30 for 13 min and 73:27 for 30 min, and the flow rate was 0.32 ml/min. The retention times of prostaglandins E_3, E_2, E_1, and D_2 were 13, 21, 27, and 31 min, respectively. Absorbance was measured at 192 nm. One-minute fractions were mixed with 4.5 ml of Formula-989 scintillation fluid (Du Pont) and the radioactivity was measured. DPM was converted to mass using the specific activity of the DHLA and EPA stock solutions. Recovery was assessed by measuring the ultraviolet absorbance of PGD_2.

Statistics

The unpaired Student's *t*-test (two-tailed) was used.

RESULTS

Similar to our previously reported results (25), the baseline fatty acid composition of EFD-1 cells was (% of total fatty acids) 38% 18:1 (n-7) plus 18:1 (n-9), 23% 16:0, 20% 18:0, 5% 16:1 (n-7), 4% 20:1 (n-9), 2% 14:0, 2% 18:3 (n-9), and 6% various n-7 and n-9 polyunsaturated fatty acids. In preliminary dose-response studies in which EFD-1 cells were incubated with either DHLA or EPA, we established that cellular incorporation of DHLA and EPA increased linearly as a function of the exogenous fatty acid concentration (data not shown). Esterification of incorporated DHLA and EPA (and fatty acid metabolites) into the cellular phospholipid pool was approximately 90% until the concentration of exogenous fatty acid was increased above 10 μM, and decreased linearly with further increases in the concentration of exogenous fatty acid, such that in EFD-1 cells incubated with 20 μM DHLA or EPA, 25–30% of incorporated fatty acid was esterified in triacylglycerol. (Neglible quantities of fatty acid entered the cholesteryl ester pool.) Therefore, in that we were interested in the competition between n-3 and n-6 fatty acids for entry into cellular phospholipids, we used a fatty acid concentration of 20 μM in all subsequent experiments. In addition, EFD-1 cells incubated with 20 μM DHLA or 20 μM EPA accumulated physiologically relevant cellular masses of n-6 or n-3 fatty acids (31 and 23 nmol/10^6 cells of n-6 and n-3 fatty acids, respectively) (32, 33). The reason for the lower incorporation of EPA compared to DHLA is unclear, but this phenomenon has been observed in other cell types (16, 34).

Time course studies

We examined the time course of DHLA and EPA uptake and metabolism by incubating EFD-1 cells with

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either 20 μM DHLA, 20 μM EPA, or 20 μM DHLA and EPA combined (40 μM total), for 3, 9, or 24 h, and then measuring the total cellular masses of incorporated fatty acids and their metabolites. **Fig. 1** represents data from the combined DHLA/EPA incubations. When cells were incubated with DHLA or EPA alone, the time course of uptake, and total fatty acid uptake, were similar to that observed when DHLA and EPA were coincubated (data not shown). As seen in Fig. 1A, fatty acid uptake increased linearly over the first 9 h of incubation and virtually ceased by 24 h. Therefore, in light of abundant evidence that the cellular distribution of incorporated fatty acids changes markedly in the first few hours of incubation and stabilizes thereafter (25, 35, 36), we sought to minimize the amount of recently incorporated fatty acid in the system by performing all subsequent incubations for 24 h. Fig. 1B shows that both DHLA and EPA accumulated rapidly early in the incubation and reached a plateau between 9 and 24 h, whereas the cellular masses of their metabolites increased throughout the incubation.

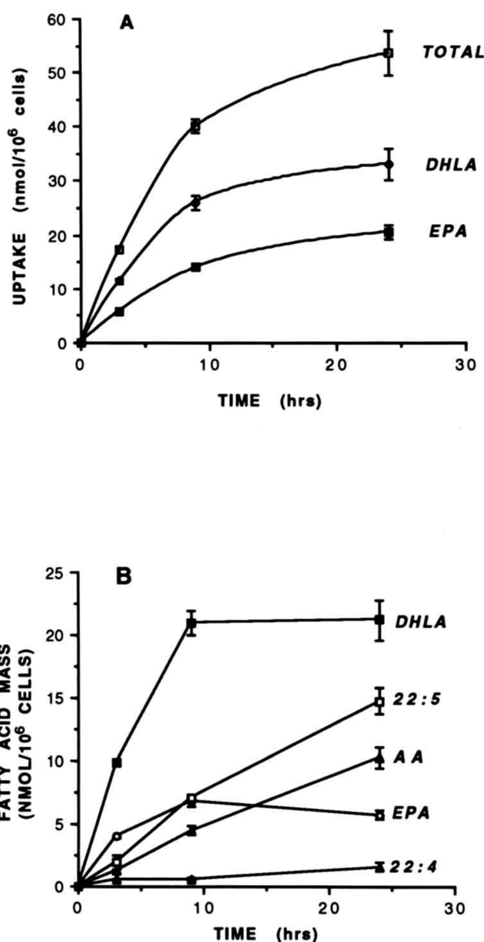

Fig. 1. Time course of DHLA and EPA uptake and metabolism. EFD-1 cells were plated in 35-mm diameter petri dishes, grown to near confluence, and then incubated for 3, 9, or 24 h with 20 μM DHLA and 20 μM EPA combined (total fatty acid concentration: 40 μM) in delipidated medium. The cells were harvested and total cellular uptake (A) and the cellular masses of DHLA, EPA, and their fatty acid metabolites (B) were measured as described in Materials and Methods. Values represent mean ± SEM of duplicate plates.

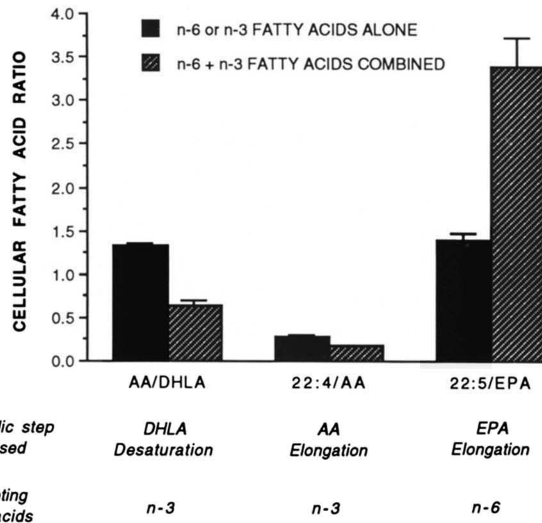

Fig. 2. Reciprocal effects of n-3 and n-6 fatty acids on DHLA, AA, and EPA metabolism. EFD-1 cells were plated in 60-mm diameter petri dishes, grown to near confluence, and then incubated for 24 h with either 20 μM DHLA, 20 μM EPA, or 20 μM DHLA and 20 μM EPA combined, in delipidated medium. The cells were harvested and the fatty acids were analyzed as described in Materials and Methods. Values represent mean ± SEM from four experiments combined (n = 3, 5, 3, 5, 5, and 5 for consecutive bars; $P < 0.003$ for each comparison between paired solid and hatched bars).

Metabolism of DHLA, AA, and EPA

We next examined the effect of n-3 fatty acids on the desaturation of DHLA to AA (AA/DHLA), and on the elongation of AA to 22:4 (22:4/AA); and the effect of n-6 fatty acids on the elongation of EPA to 22:5 (22:5/EPA). As can be seen in **Fig. 2,** n-3 fatty acids significantly inhibited the desaturation of DHLA to AA and the elongation of AA to 22:4. In contrast, n-6 fatty acids markedly stimulated the elongation of EPA to 22:5. It is also of interest that elongation of EPA was far more extensive than elongation of AA. At the concentration of EPA used in these experiments, docosahexaenoic acid (DHA, 22:6 Δ4,7,10,13,16,19), the Δ4 desaturation product of 22:5, was not detected. In preliminary dose-response experiments in which EFD-1 cells were incubated with EPA alone for 24 h, DHA constituted approximately 8% and 4% of total cellular n-3 fatty acids at exogenous EPA concentrations of 5 and 10 μM, respectively. The higher conversion of 22:5 to DHA when lower concentrations of exogenous EPA were used may have been due to the residual essential fatty acid deficiency of the cells at these concentrations, as Δ4 desaturase activity has been reported to be increased in essential fatty acid deficiency (2).

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Phospholipid/triacylglycerol partitioning of DHLA, AA, and EPA

Abundant evidence indicates that DHLA, AA, and EPA compete for esterification into cellular phospholipids, and that excess cellular fatty acids enter cellular lipid storage pools, predominantly triacylglycerol (2). As shown in **Fig. 3,** addition of the second fatty acid to the incubation medium decreased the fraction of each cellular fatty acid that was esterified in phospholipid (PL) relative to triacylglycerol (TG). This decrease in the PL:TG ratio was expected because coincubation of DHLA and EPA doubled the total concentration of fatty acid in the incubation medium and, in that uptake of DHLA and EPA was additive, the total mass of incorporated fatty acid significantly increased. However, the PL:TG ratio of DHLA, AA, 22:4, and 22:5 decreased to a relatively similar extent (2.4-, 2.3-, 4.0-, and 2.7-fold reductions, respectively), whereas the PL:TG ratio of EPA decreased 9.4-fold. In order to further demonstrate that the marked shift of EPA from phospholipid to triacylglycerol was due specifically to the addition of n−6 fatty acids rather than a nonspecific effect of the increase in the total polyunsaturated fatty acid concentration, we compared the reduction in the PL:TG ratio of EPA when 20 μM DHLA was added to the 20 μM EPA, with the reduction in the same ratio when an additional 20 μM EPA was added (40 μM total EPA concentration). In contrast to the 9.4-fold reduction in the PL:TG ratio of EPA induced by addition of 20 μM DHLA, addition of 20 μM EPA caused only a 3.6-fold reduction. On the other hand, 22:5 shifted from phospholipid to triacylglycerol in response to additional DHLA or EPA to the same extent (2.5- vs. 2.7-fold reductions in PL:TG). We next compared the cellular PL:TG ratio of DHLA and AA when cells were incubated with 20 μM DHLA alone, 40 μM DHLA, or 20 μM DHLA and 20 μM EPA (40 μM total fatty acid concentration). Increasing the total fatty acid concentration to 40 μM with either additional DHLA or EPA induced similar reductions in the PL:TG ratio of both DHLA and AA (data not shown). Thus, by both stimulating the elongation of EPA to 22:5 and causing a specific shift in cellular EPA from phospholipids to triacylglycerol, n−6 fatty acids markedly limited the accumulation of EPA in the phospholipid pool. Indeed, when EFD-1 cells were incubated with EPA alone at concentrations which ranged from 5 to 40 μM, the mass of EPA in cellular phospholipids increased more than 3-fold as the concentration EPA increased. In contrast, when DHLA and EPA were coincubated at equimolar concentrations ranging from 5 to 40 μM, the mass of EPA in phospholipids remained unchanged as the concentration of exogenous EPA increased (data not shown).

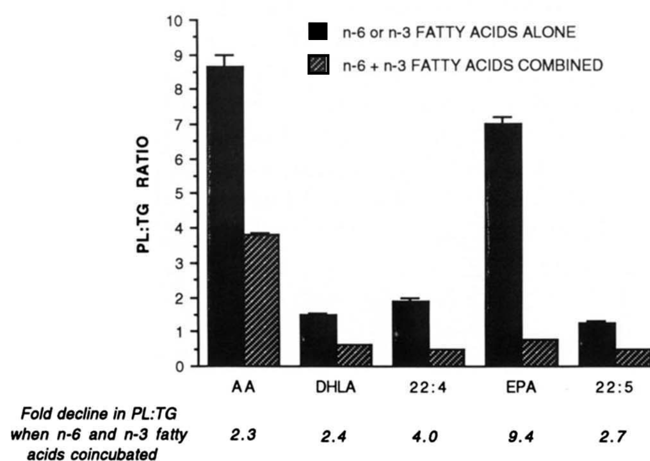

Fig. 3. Reciprocal effects of n−3 and n−6 fatty acids on phospholipid-triacylglycerol partitioning of DHLA, AA, and EPA. EFD-1 cells were plated in 60-mm diameter petri dishes, grown to near confluence, and then incubated for 24 h with either 20 μM DHLA, 20 μM EPA, or 20 μM DHLA and 20 μM EPA combined, in delipidated medium. The cells were harvested and the fatty acids were analyzed as described in Materials and Methods. Values represent data from three experiments (mean ± SEM of duplicates; $P < 0.005$ for each comparison).

Phospholipid distribution studies

In order to further evaluate the reciprocal effects of n−6 and n−3 fatty acids on fatty acid esterification into phospholipids, we incubated EFD-1 cells with either DHLA or EPA alone, or DHLA and EPA combined, as before, and then analyzed by mass the total fatty acid content of the cellular diacyl phospholipids and the *sn*-2 position (acyl linkage) of the plasmalogens. Eicosanoid precursor fatty acids have been shown to be present almost exclusively in the *sn*-2 position of plasmalogens (37). In these experiments, we did not separate the diacyl from the 1-O-alkyl-2-acyl forms of the choline-containing and ethanolamine-containing glycerophospholipids. **Fig. 4A** shows the masses of AA, DHLA, EPA, and 22:5 in the total cellular phospholipid pool (PL) and in the individual phospholipid species, both in the absence (solid bars) and presence (hatched bars) of the second family of fatty acids. (In the presence of n−3 fatty acids, the mass of 22:4 in individual cellular phospholipid species was below the level required for reproducible quantitation.) The amount of phosphatidylserine in EFD-1 cells has been previously shown to be extremely small (25), and in these experiments phosphatidylserine did not incorporate any n−6 or n−3 fatty acids. As can be seen, addition of n−3 fatty acids led to an approximately 25% decrease in the mass of AA in the total cellular phospholipid pool. However, only phosphatidylinositol (PI) showed a statistically significant loss of AA mass (36% reduction, $P = 0.0008$). The mass of AA in phosphatidylcholine decreased slightly, but this decrease did not reach statistical significance ($P = 0.10$). Similarly, competition with n−3 fatty acids significantly decreased the mass of DHLA only in PI (45% reduction, $P = 0.005$). In contrast, competition with n−6 fatty acids resulted in a marked (63%) reduction in the mass of EPA

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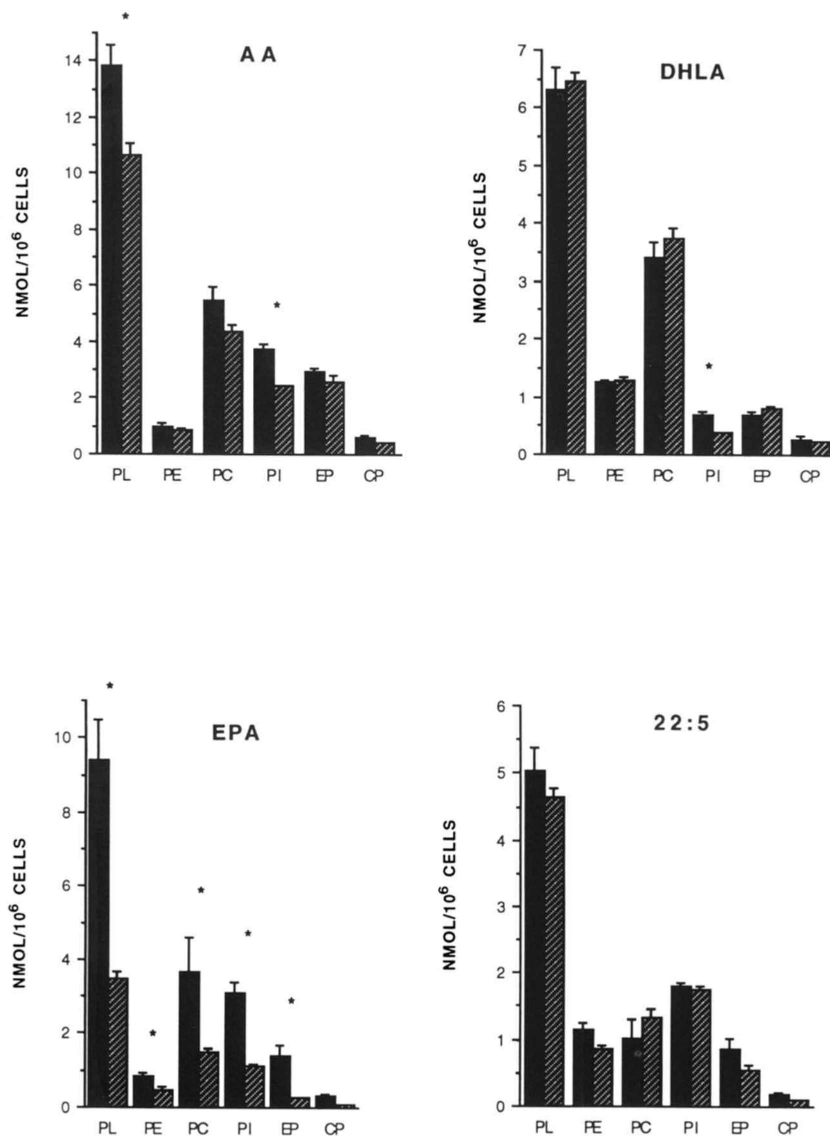

Fig. 4. Reciprocal effects of n-3 and n-6 fatty acids on incorporation of AA, DHLA, EPA, and 22:5 into cellular phospholipids. EFD-1 cells were plated in 100-mm diameter petri dishes, grown to near confluence, and then incubated for 24 h with either 20 μM DHLA, 20 μM EPA, or 20 μM DHLA and 20 μM EPA combined, in delipidated medium. The cells were harvested and the fatty acids were analyzed as described in Materials and Methods. Solid bars represent incubations with n-6 or n-3 fatty acids alone. Hatched bars represent incubations with n-6 and n-3 fatty acids combined. Values include data from three experiments (mean ± SEM, n = 3 or 4 for all bars except for hatched EP (2) and hatched CP (1)). The abbreviations, PL, EP, and CP, represent total cellular phospholipids, ethanolamine plasmalogen, and choline plasmalogen, respectively. Asterisks denote statistical significance ($P < 0.05$).

from the total cellular phospholipid pool, but this reduction involved all cellular phospholipids to a relatively similar extent. Despite the marked changes observed with EPA, the mass of 22:5 did not change significantly in either the total phospholipid pool or in the individual phospholipids. Of note, in the absence of competing fatty acids, PI was selectively enriched in EPA, 22:5, and AA. Thus, whereas PI constituted only 11% of total phospholipids, 34%, 37%, and 27% of total phospholipid-associated EPA, 22:5, and AA, respectively, were esterified in PI. In contrast, only 11% of phospholipid-associated DHLA was esterified in PI. This analysis further demonstrated the specificity of the effect of n-3 fatty acids on the incorporation of DHLA and AA to PI. In the presence of n-3 fatty acids, the percentage of total phospholipid-associated AA and DHLA was reduced only in PI (to 23% and 6%, respectively, $P < 0.004$ for each reduction), whereas in the presence of n-6 fatty acids, the mass distribution of EPA and 22:5 remained essentially unchanged.

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Prostaglandin release studies

Finally, we determined the effect of n−3 fatty acids on the agonist-induced synthesis of PGE_1 and PGE_2, and the effect of n−6 fatty acids on the synthesis of PGE_3. EFD-1 cells were incubated with either 20 μM [^{14}C]DHLA, 20 μM [^{14}C]EPA, or 20 μM of [^{14}C]DHLA and [^{14}C]EPA combined, and then stimulated with bradykinin. Released ^{14}C-labeled PGE_1, PGE_2, and PGE_3, derived from DHLA, AA, and EPA, respectively, were separated by HPLC and their masses were calculated from measured radioactivity. We have previously shown that under these experimental conditions, minimal conversion of PGE to PGA or PGB occurs (38). As seen in **Fig. 5,** addition of EPA to the incubation medium caused a 33% reduction in the agonist-induced release of both PGE_1 and PGE_2. This reduction was similar in magnitude to the reduction of the masses of AA and DHLA in PI (36% and 45%, respectively). Fig. 5 also shows that the small amount of PGE_3 that was produced by EFD-1 cells when incubated with EPA alone increased 2.3-fold in the presence of n−6 fatty acids (P = 0.002). This seemingly paradoxical effect of n−6 fatty acids has been noted previously, and is likely due to activation of cyclooxygenase either by oxygenated metabolites of n−6 fatty acids or by n−6 fatty acids themselves (3, 39, 40).

DISCUSSION

We have studied the interactions between n−6 and n−3 fatty acids in an essential fatty acid-deficient cell line, EFD-1. This cell line provided a well-controlled system for quantitative analysis of cellular fatty acid metabolism, and, although by definition EFD-1 cells have an abnormal baseline fatty acid profile, evidence suggests that results of metabolic studies in these cells can be extrapolated to cells with a normal fatty acid content. First, the phospholipid distribution of arachidonate in EFD-1 cells supplemented with n−6 fatty acids is similar to that of the parent cell line (25), and the kinetics of phospholipid turnover of arachidonate recently incorporated into EFD-1 cells is similar to that reported in cell types with a normal fatty acid profile (36, 41). Second, essential fatty acid depletion in EFD-1 cells does not disrupt the cellular mechanisms of agonist-stimulated prostaglandin production, in that repletion of EFD-1 cells with arachidonate restores agonist-stimulated PGE_2 production to levels comparable to that of the parent cell line (25). Third, the morphologic alterations in EFD-1 cells induced by essential fatty acid deficiency are reversed by repletion with arachidonate (42). In addition to further characterizing the well-studied effects of n−3 fatty acids on AA metabolism, we were able in this study to analyze interactions about which information is scant, such as the interactions between DHLA and EPA, and the specific effects of n−6 fatty acids on EPA metabolism. Our results include the findings that *1)* n−6 fatty acids markedly stimulated the elongation of EPA to 22:5; *2)* n−6 fatty acids caused a specific shift of cellular EPA from phospholipid to triacylglycerol; *3)* n−3 fatty acids caused a specific reduction of AA and DHLA mass in phosphatidylinositol, whereas n−6 fatty acids caused a marked reduction of EPA mass in all cellular phospholipids; and *4)* n−3 fatty acids inhibited PGE_1 production, and this inhibition was identical in magnitude to the inhibition of PGE_2 production.

Desaturation and elongation of n−6 and n−3 fatty acids are critically influenced by the activities of a variety of cellular fatty acid desaturases, elongases, and retroconverting enzymes (2, 43). Although our experiments do not specifically address the enzymatic mechanisms that underlie these reactions, they generated a number of interesting results. Desaturation of DHLA to AA and elongation of AA to 22:4 were inhibited by n−3 fatty acids; and elongation of EPA to 22:5 was markedly stimulated by n−6 fatty acids. In addition, as in other cell types (44, 45), elongation of EPA was far more extensive under all conditions than elongation of AA. The inhibitory effect of n−3 fatty acids on AA elongation has been demonstrated in cell culture (44), but stimulation of EPA elongation by n−6 fatty acids has not previously been observed. The high AA:22:4 and low EPA:22:5 ratios which have been observed in most tissues (46, 47) are strong evidence that the metabolism of n−3 and n−6 fatty acids in vivo is regulated in a similar fashion.

In addition to reducing the cellular mass of EPA by stimulating its metabolism, n−6 fatty acids caused a profound shift of EPA from cellular phospholipids to triacylglycerol, thus markedly reducing the amount of EPA that entered the phospholipid pool (Fig. 4A). The mechanism

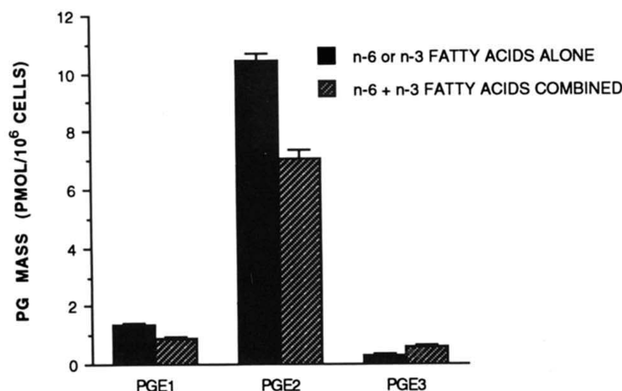

Fig. 5. Reciprocal effects of n−3 and n−6 fatty acids on PGE_1, PGE_2, and PGE_3 production. EFD-1 cells were plated in 60-mm diameter petri dishes, grown to near confluence, and then incubated with 20 μM [^{14}C]DHLA, 20 μM [^{14}C]EPA, or 20 μM of [^{14}C]DHLA and [^{14}C]EPA combined, for 24 h. The cell monolayers were stimulated with bradykinin and released prostaglandins were analyzed as described in Materials and Methods. Values include data from two experiments combined (mean ± SEM, n = 4 for PGE_1 and PGE_2 values; n = 5 for PGE_3 values; $P < 0.005$ for each comparison).

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that underlies this specific effect of n-6 fatty acids on EPA is uncertain, but evidence from our laboratory suggests that competition for arachidonoyl-CoA synthetase is important in the regulation of the PL:TG partitioning of polyunsaturated fatty acids (38). In support of the hypothesis that competition for arachidonoyl-CoA synthetase at least partly explains our results, excess EPA (as opposed to DHLA or AA) only weakly inhibited the formation of arachidonoyl-CoA from labeled AA by human platelet arachidonoyl-CoA synthetase (relative K_i for AA, DHLA, and EPA: 1.0, 3.9, and 9.2, respectively) (48). In addition, rapid uptake of AA into endothelial cell phospholipids, which is believed to be mediated primarily by arachidonoyl-CoA synthetase, was inhibited 2.5-fold by competing EPA, whereas rapid uptake of EPA was inhibited 5.1-fold by competing AA (49). From a therapeutic standpoint, the observations that n-6 fatty acids stimulated EPA elongation and induced a redistribution of cellular EPA may explain both the difficulty in achieving high levels of EPA in cellular phospholipids when the diet is supplemented with EPA (6, 14, 50), and the high doses of dietary EPA that are needed in order to achieve a physiologic response (11). In addition, these findings suggest that dietary therapies designed to increase the EPA content of tissue phospholipids may need to focus on limiting n-6 fatty acid intake in addition to increasing EPA intake.

As shown in Fig. 5, when incubated with n-6 fatty acids alone, EFD-1 cells produced PGE_2 far in excess of PGE_1. We have previously shown that this is due to selective release of AA over DHLA from the total cellular phospholipid pool, and that this selective release is likely due to the selective incorporation of AA into PI and ethanolamine plasmalogen (38). Addition of n-3 fatty acids to the system caused a 33% reduction of PGE_1 and PGE_2 release. Evidence that PI is the major phospholipid donor of polyunsaturated fatty acids for eicosanoid synthesis in EFD-1 cells (51, 52), together with our observations that *1)* n-3 fatty acids significantly reduced AA and DHLA mass only in PI, and *2)* this reduction was similar in magnitude to the reduction of PGE_1 and PGE_2 release, suggests that n-3 fatty acids reduced PGE_1 and PGE_2 release by reducing the mass of their precursor fatty acids in PI. However, we cannot exclude the possibility that the reduction in PGE_1 and PGE_2 production was at least partially due to competitive inhibition of cyclooxygenase by simultaneously released EPA. The finding that n-3 fatty acids reduced the one-series prostaglandin production is of interest in light of the documented salutary effect of DHLA and PGE_1 on a number of inflammatory processes (4, 5, 20, 21). To the extent that the anti-inflammatory effects of DHLA are mediated by increases in endogenous PGE_1 production, dietary EPA could inhibit these beneficial effects. On the other hand, numerous reports indicate that, in addition to its effects on a variety of cardiovascular processes, EPA also has potent anti-inflammatory effects, and it is believed that entry of EPA into cellular phospholipids is necessary in order to achieve these effects (6, 7, 50). For example, cellular production of leukotriene B_5, the functionally attenuated EPA-derived analog of the AA-derived leukotriene B_4, has been shown to be closely correlated with the level of EPA in cellular phospholipids; and it has been postulated that an increase in the ratio of leukotriene B_5 to leukotriene B_4 in tissues may contribute to the anti-inflammatory effects of EPA (15, 18). Our data, which show that the mass of EPA in cellular phospholipids was markedly reduced in the presence of DHLA, are clearly important in this regard. This issue is further complicated by the observation that, unlike the generation of lipoxygenase products of EPA, generation of cyclooxygenase products of EPA can increase in the presence of n-6 fatty acids (Fig. 5) (39, 40). It is clear, then, that in order to effectively integrate DHLA and EPA into future dietary therapies, a better understanding is needed both of the mechanisms that underlie their biological effects and of their complex metabolic interactions in vivo.

This work was supported by Grant No. DK 43159 from the National Institutes of Health.

Manuscript received 2 November 1991 and in revised form 11 March 1992.

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