Eicosapentaenoic acid metabolism in brain microvessel endothelium: effect on prostaglandin formation

Nagender R. Yerram, Steven A. Moore,[1,*] and Arthur A. Spector

Departments of Biochemistry and Pathology,* The University of Iowa, Iowa City, IA 52242

Abstract Mouse brain microvessel endothelial cells convert eicosapentaenoic acid (EPA) to prostaglandin (PG) E_3, PGI_3, and several hydroxy fatty acid derivatives. Similar types of products are formed by these microvessel endothelial cells from arachidonic acid. The formation of PGI_2 and PGE_2 is reduced, however, when the brain microvessel endothelial cultures are incubated initially with EPA. Exposure to linolenic or docosahexaenoic acid also decreased the capacity of these microvessel endothelial cells to form PGI_2 and PGE_2, but the reductions were smaller than those produced by EPA. Like the endothelial cultures, intact mouse brain microvessels convert EPA into eicosanoids, and incubation with EPA reduces the subsequent capacity of the microvessels to produce PGI_2 and PGE_2. Brain microvessel endothelial cells took up less EPA than arachidonic acid, primarily due to lesser incorporation into the inositol, ethanolamine, and serine glycerophospholipids. By contrast, considerably more EPA than arachidonic acid was incorporated into triglycerides. These findings suggest that the microvessel endothelium may be a site of conversion of EPA to eicosanoids in the brain and that EPA availability can influence the amount of dienoic prostaglandins released by the brain microvasculature. Furthermore, the substantial incorporation of EPA into triglyceride suggests that this neutral lipid may play an important role in the processing and metabolism of EPA in brain microvessels. —**Yerram, N. R., S. A. Moore, and A. A. Spector.** Eicosapentaenoic acid metabolism in brain microvessel endothelium: effect on prostaglandin formation. *J. Lipid Res.* 1989. **30:** 1747–1757.

Supplementary key words arachidonic acid • prostacyclin • prostaglandin E_2 • triglycerides

Eicosapentaenoic acid (20:5 (n-3), EPA), a member of the omega-3 series of polyunsaturated fatty acids, appears to protect against coronary heart disease, ischemic brain injury, thrombosis, and some inflammatory diseases (1–5). One of the effects of EPA is to decrease the conversion of arachidonic acid to thromboxane A_2 in platelets (6) and to leukotriene B_4 in neutrophils (7). This reduces platelet aggregation and the inflammatory response, effects that probably are responsible for many of the protective actions of EPA.

In addition to its effects on platelets and neutrophils, EPA has been observed to reduce prostaglandin formation in other types of cells, i. e., vascular smooth muscle, macrophages, and 3T3 fibroblasts (8–10). A similar effect has been observed in human umbilical vein, bovine aortic, and bovine pulmonary artery endothelial cell cultures (10–12). EPA reduces prostacyclin (PGI_2) production, but itself is not readily utilized for prostaglandin formation in these endothelial cultures. Studies with cyclooxygenase preparations also indicate that EPA is a poor substrate for this enzyme (13). Recent clinical findings, however, are not consistent with the conclusions drawn from the studies with endothelial cultures. In the clinical investigations, prostaglandin formation was assessed in patients fed n-3 fatty acid supplements. Analysis of urinary metabolites by mass spectrometry indicated that PGI_2 formation was not reduced by the dietary n-3 fatty acids and, furthermore, that substantial amounts of EPA were converted to PGI_3, a PGI_2 analogue that also has anti-aggregatory properties (14, 15).

A possible explanation for the differences between the clinical and cell culture data may be that the large vessel endothelia studied in vitro are not representative of the endothelial cells that contribute the bulk of the PGI_2 measured in vivo. To assess this possibility, we have examined the effects of EPA on microvascular endothelium. These endothelial cells, which have been cultured from murine cerebral microvessels, produce sizable amounts of PGI_2 (16). In the present work we have investigated the utilization of EPA by

Abbreviations: EPA or 20:5, eicosapentaenoic acid; FBS, fetal bovine serum; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; HPLC, high performance liquid chromatography; PGE_2, prostaglandin E_2; PGI_1, prostacyclin (prostaglandin I_2); HHT, 12-hydroxyheptadecatrienoic acid; TLC, thin-layer chromatography; 16:0, palmitic acid; 18:2, linioleic acid; 18:3, linolenic acid; 20:4, arachidonic acid; 22:6, docosahexaenoic acid; PBS, phosphate-buffered saline.

[1]Address for correspondence: Department of Pathology, The University of Iowa, Iowa City, IA 52242

the microvessel endothelial cultures and determined whether exposure to EPA or other n-3 fatty acids influence the capacity of these cells to produce PGI_1.

METHODS

Cell culture

Murine cerebromicrovascular endothelial cells were isolated from mouse brains as described previously (16). Briefly, the brains of mouse pups were aseptically removed under general anesthesia and disrupted in a Dounce homogenizer. Microvessels were collected either on nylon mesh (150 μm) or glass beads after dextran fractionation and plated in plastic tissue culture dishes. Endothelial cells migrating from vessels were pooled to form a proliferating cell line. The cultures were maintained in Lewis medium containing 10% fetal bovine serum (FBS, Sigma, St. Louis, MO). The purity and characterization of endothelial cells were established by light and electron microscopic appearance, the presence of thrombomodulin activity, and *Griffonia simplicifolia* agglutinin (Sigma) histochemical staining (17). Contamination of cultures by smooth muscle and astrocytes was determined by immunohistochemical staining with anti-α-actin antibody, HUC 13 (18), and anti-glial fibrillary acid protein antibodies, respectively. The cultures were found to contain 90–98% endothelial cells, with the contamination accounted for by smooth muscle cells. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The cells were subcultured weekly by trypsinization, and cells obtained between passages 8 and 18 were seeded into 12-well plates (100 to 120 μg total cell protein/well) for each experiment.

Human umbilical vein endothelial cells were isolated and cultured as described previously (11). Briefly, the cells were enzymatically removed from human umbilical veins, suspended in modified Medium 199 containing 20% FBS, and seeded at 2.25×10^6 cells per well in 6-well plates. After 24 h, this medium was replaced by modified Medium 199 containing 25 mM HEPES plus 20% FBS. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. All experiments were performed on confluent primary cultures.

Microvessel isolation

Mouse brain microvessels were isolated from adult Swiss-Webster mouse brains after transcardiac perfusion with 10 units/ml heparinized normal saline to remove blood elements. After disrupting the brains in a Dounce homogenizer, the microvessels were isolated by fractionation with 15% dextran. During perfusion and microvessel isolation, all solutions contained 10 μM ibuprofen to prevent inactivation of cyclooxygenase. The microvessels were collected on nylon mesh filters and thoroughly washed with ibuprofen-free Hanks buffered salt solution. After transfer to a tissue culture dish, the isolated microvessels were incubated with experimental medium.

Incubations

The isolated brain microvessels or cultured microvessel endothelial cells (90–95% confluent) were washed with serum-free Lewis medium and incubated with medium containing 1% FBS supplemented with fatty acids. Either 1 μCi/ml [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (200 Ci/mmol, Research Products Intl. Mount Prospect, IL), [5,6,8,9,11,12,14,15-^{3}H]eicosapentaenoic acid (79.0 Ci/mmol, New England Nuclear, Boston, MA), or nonradioactive fatty acids were added to the medium as a warm solution of the sodium salt. The nonradioactive fatty acids were obtained from Nu-Chek-Prep (Elysian, MN). After incubation with the fatty acid-supplemented medium for the indicated period of time, the medium was removed and the cells were washed with cold PBS containing 10 μM BSA to remove adherent fatty acids. The cells then were harvested by rapid addition of 1 ml of cold methanol–acetic acid 99:1(v/v) followed by scraping with a rubber policeman.

Lipid extraction and thin-layer chromatography

The cells were scraped into a total volume of 3 ml cold methanol–acetic acid 99:1(v/v), and the lipid was extracted with 6 ml chloroform and 2 ml 0.15 M NaCl containing 4 mM HCl, a modification of the method of Folch, Lees, and Sloane Stanley (19). After vortexing, the phases were separated by centrifugation at 1000 *g* for 10 min. The lower phase was collected and the upper phase was re-extracted with 5 ml of chloroform–methanol–0.15 M NaCl containing 4 mM HCl 84:14:1(v/v/v). The combined chloroform extracts were dried under N_2 and redissolved in chloroform–methanol 1:1(v/v). Aliquots of the lipid extract were assayed in vials containing 4 ml of Budget Solve scintillation solution (Research Products Intl.) for determination of total lipid radioactivity using a Packard Tri-carb 460 liquid scintillation spectrometer. Quenching was monitored with a ^{226}Ra external standard. Other aliquots of the extracted cell lipids were chromatographed on LK-5D TLC plates (Whatman, Clifton, NJ) with a solvent system containing chloroform–methanol–water–acetic acid 50:50:1.5:2 (v/v/v/v). Phospholipids and neutral lipid standards were added, and the chromatogram was visualized under UV light after spraying 1 mM 8-anilino-1-naphthalenesulfonate. The distribution of radioactivity on the TLC plate was determined with a TLC scanner Model R (Radiomatic Instruments & Chemical Co., Tampa, FL).

High performance liquid chromatography

Confluent endothelial cultures were washed with serum-free medium and then incubated with either 1 μCi/ml [^{3}H]arachidonic acid or [^{3}H]eicosapentaenoic acid in se-

rum-free medium. After incubation for 20 min, the medium was collected and centrifuged to remove cellular debris. In additional experiments, the cells initially were incubated with labeled arachidonic acid or EPA for 24 h. After this medium was removed, the cells were washed and then incubated with medium containing 2 μM ionophore A23187 for 20 min. Lipids were extracted from all of the media with chloroform–methanol 2:1(v/v) containing 1% acetic acid. The chloroform phase was dried under N_2 and resuspended in acetonitrile–water 1:1.5(v/v) adjusted to pH 3.4 with phosphoric acid. Aliquots of lipids were separated on a Beckman 332 HPLC gradient system equipped with a C_{18} reverse-phase column containing 5 μm spherical packing. The elution gradient consisted of water adjusted to pH 3.5 with phosphoric acid and acetonitrile; the starting gradient mixture contained 30% acetonitrile and increased to 100% over 45 min by programming (20). Radioactivity was monitored by mixing the column effluent with Budget Solve scintillation solution at 1:3 (v/v) and passing the mixture through an on-line Flo-one/β radioactivity detector (Radiomatic Instruments & Chemical Co.). The elution profile of the system was characterized with the following standards; 6-keto prostaglandin $F_{1\alpha}$ (6 keto $PGF_{1\alpha}$), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin E_2 (PGE_2), leukotriene B_4 (LTB_4), 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT), 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), 12-hydroxy-6,8,11,14-eisosatetraenoic acid (12-HETE), 15-hydroxy-6,8,11,14-eicosatetraenoic acid (15-HETE), and arachidonic acid.

Radioimmunoassay

After the confluent endothelial cultures were incubated with fatty acid-supplemented medium, this medium was removed, and the monolayers were washed and then incubated with serum-free medium containing 7.5 μM arachidonic acid, 100 μM acetylcholine or 2 μM ionophore A23187 for 10 or 20 min. The medium was collected and centrifuged, and aliquots of the supernatant solution were assayed for 6-keto $PGF_{1\alpha}$, the stable catabolic product of PGI_2, and PGE_2 by radioimmunoassay. Specific antibodies were obtained from Advanced Magnetics Inc. (Cambridge, MA); radiolabeled prostaglandins were from New England Nuclear; and prostaglandin standards from Caymen Chemicals (Ann Arbor, MI). Aliquots (100 μl) of samples or standards were incubated for 30 min with 50 μl of the appropriate antibody. After a further 16 h incubation with 50 μl of radiolabeled prostaglandin, 400 μl of 1% dextran-coated charcoal was added to each tube, and the contents were centrifuged for 10 min at 4°C. A 500-μl aliquot of the supernatant solution was added to 4.5 ml Budget Solve scintillation solution and the radioactivity was measured in a Beckman LS 2800 scintillation spectrometer. A complete standard curve was run with each assay; the detection limits were 0.25–50 pmol/ml. The 6-keto-$PGF_{1\alpha}$ antibody has a 7.8% cross-reactivity with $PGF_{1\alpha}$, 6.8% with 6-keto-PGE_1, 2.2% with $PGF_{2\alpha}$, and <1.0% with all other prostaglandins. The PGE_2 antibody has a 100% cross-reactivity with PGE_1, 6.0% with PGA_2, 3.0% with PGA_1, 1.3% with $PGF_{2\alpha}$, and <1.0% with all other prostaglandins.

Phospholipid assay

The phosphorus content of the extracted cell lipids was determined colorimetrically by the procedure of Chalvardjian and Rudnicki (21) using dipalmitoyl phosphatidylcholine as standard.

RESULTS

Eicosanoid production

Fig. 1 illustrates the HPLC profiles of the radioactive eicosanoids released into the medium when the mouse brain microvessel endothelial cultures were labeled with either

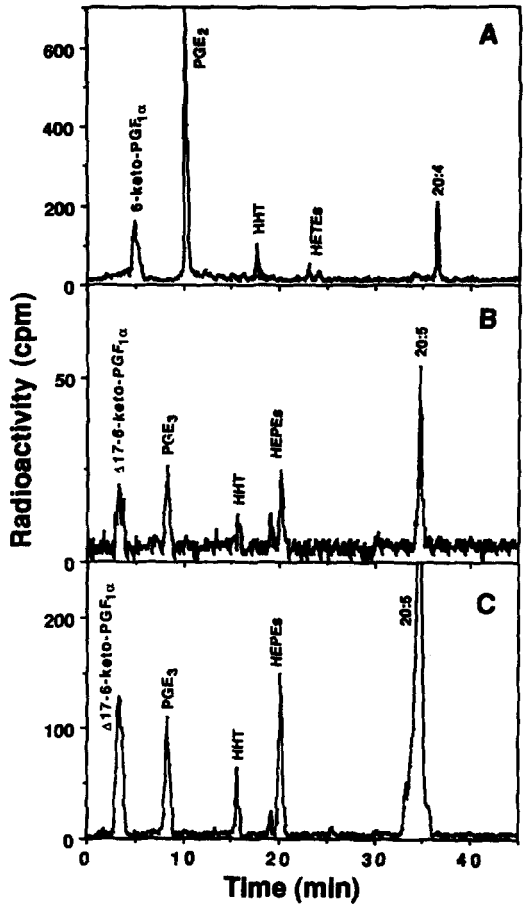

Fig. 1. Radioactive eicosanoids formed by microvessel endothelial cell cultures. Representative HPLC chromatograms of the radioactive eicosanoids produced by microvessel endothelial cells during a 20-min incubation with 2 μM calcium ionophore A23187 after labeling of the cell lipids for 24 h with either [^{3}H]arachidonic acid (A) or [^{3}H]EPA (B). Similar metabolites were produced during a direct 20-min incubation with 7.5 μM [^{3}H]EPA (C).

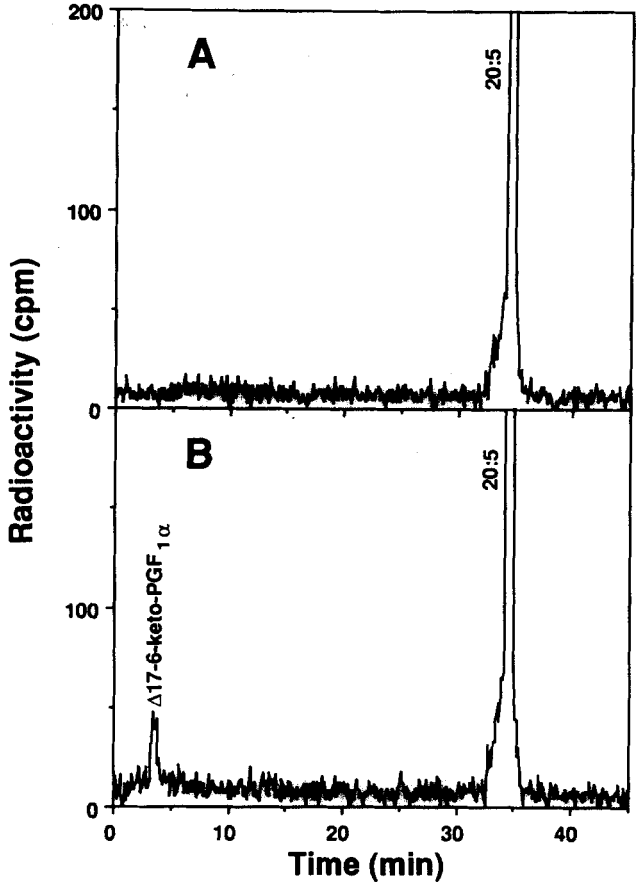

Fig. 2. Radioactive eicosanoids formed by human umbilical vein endothelial cell cultures. Representative HPLC chromatograms of the radioactive eicosanoids produced by the human umbilical vein endothelial cells during a 20-min incubation with 7.5 μM [^{3}H]EPA (A) or with 7.5 μM [^{3}H]EPA plus 20 μM arachidonic acid (B).

arachidonic acid or EPA and then stimulated with the calcium ionophore A23187. The 2–10% smooth muscle content of these cultures is unlikely to have contributed significantly to these profiles, since the eicosanoid production of endothelial cell cultures highly purified by fluorescence-activated cell sorting is unchanged from their prepurified production (17). The main prostaglandin produced by ionophore-stimulated microvessel endothelial cell cultures of the present study was PGE_2 (Fig. 1A). Some 6-keto-$PGF_{1\alpha}$, the inactivation product of PGI_2, and unmodified arachidonic acid were also detected. In addition, small amounts of radioactivity with retention times similar to HHT and HETEs were present. As shown in Fig. 1B, the largest radioactive product released when the microvessel endothelial cultures labeled with EPA were exposed to ionophore A23187 was unmodified fatty acid. However, radioactive metabolites with retention times corresponding to Δ17-6-keto-$PGF_{1\alpha}$, the inactivation product PGI_3, and PGE_3 were observed, as well as several additional radioactive components having the retention times of 12-hydroxyheptadecatetraenoic acid and hydroxyeicosapentaenoic acids (HEPEs).

The radioactive metabolites produced when cultured mouse brain microvessel endothelial cells were incubated for 20 min with [^{3}H]EPA are shown in Fig. 1C. The metabolites formed were similar to those released when the cells labeled with EPA were incubated with the ionophore, with the major components again being Δ17-6-keto-$PGF_{1\alpha}$, PGE_3, and several hydroxy-derivatives.

Fig. 2 illustrates the HPLC profiles of the radioactive eicosanoids released into the medium when human umbilical vein endothelial cells were incubated with either 7.5 μM [^{3}H]EPA or 7.5 μM [^{3}H]EPA plus 20 μM arachidonic acid. No EPA metabolites were detected when the cells were incubated with 7.5 μM [^{3}H]EPA alone. However, a single radioactive peak eluting 2.5 min earlier than 6-keto-$PGF_{1\alpha}$ was detected when the cells were concomitantly incubated with 7.5 μM [^{3}H]EPA and 20 μM arachidonic acid. Bordet,

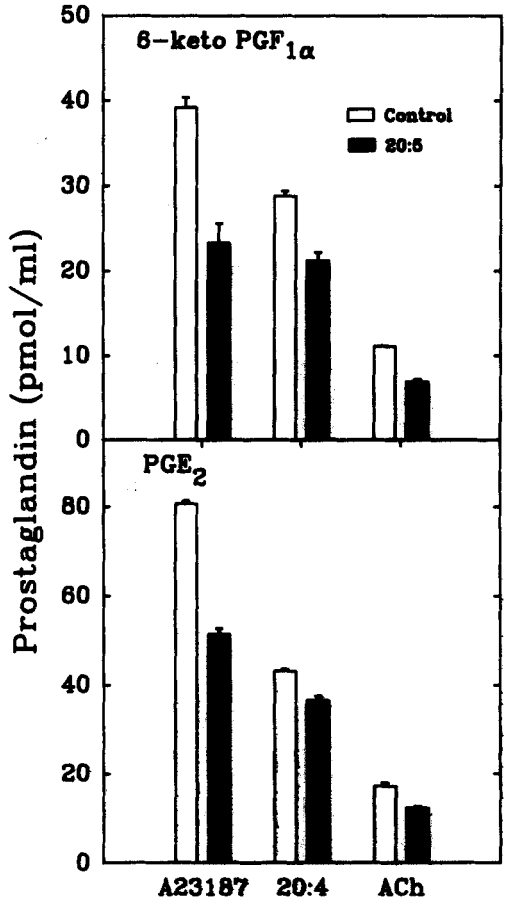

Fig. 3. Prostaglandin I_2 and E_2 production by microvessel endothelium. Microvessel endothelial cells were incubated with Lewis medium containing 1% FBS supplemented with 20 μM EPA for 10 h. After removal of the supplemental medium and washing, the cells were incubated with serum-free medium containing 7.5 μM arachidonic acid, 100 μM acetylcholine or 2 μM ionophore A23187 for 20 min. The medium was collected and centrifuged and aliquots of the supernatant were assayed for 6-keto-$PGF_{1\alpha}$ (top) and PGE_2 (bottom) by radioimmunoassay. Each bar is the mean ± SE of values obtained from three separate cultures.

Guichardant, and Lagarde (22) identified this EPA metabolite by gas–liquid chromatography–mass spectrometry as Δ17-6-keto-$PGF_{1\alpha}$, the inactivation product of PGI_3, in similar incubations with human umbilical vein endothelial cells.

Effect of EPA on microvessel endothelial dienoic prostaglandin production

Previous studies indicate that exposure to EPA reduces the capacity of human and bovine endothelium derived from large vessels to produce PGI_2 (11, 12). A similar response to EPA was observed in the microvessel endothelial cultures. As seen in **Fig. 3**, less PGI_2 and PGE_2 was produced by microvessel endothelial cells initially exposed for 10 h to 20 μM EPA, as compared to corresponding controls incubated for a similar period without EPA. Prostaglandin production was decreased when the cells were incubated with calcium ionophore A23187, arachidonic acid, or acetylcholine. The reductions in PGI_2 formation ranged from 23 to 39%; those for PGE_2 from 12 to 37%.

The time-dependence of the EPA effect is shown in **Fig. 4A**. Microvessel endothelial cultures were incubated with 20 μM EPA for various lengths of time prior to exposure to ionophore A23187. Less PGI_2 was formed by the cells incubated with EPA than by corresponding control cultures incubated for the same period of time without supplemental EPA. The reduction in PGI_2 formation became greater as the time of incubation increased, up to 10 h. The maximum reduction produced by EPA in this experiment was 52%.

As seen in Fig. 4B, the extent of the reduction in ionophore A23187-stimulated PGI_2 formation was also dependent on the concentration of supplemental EPA. The maximum decrease observed in this experiment, 60%, was produced by 30 μM EPA.

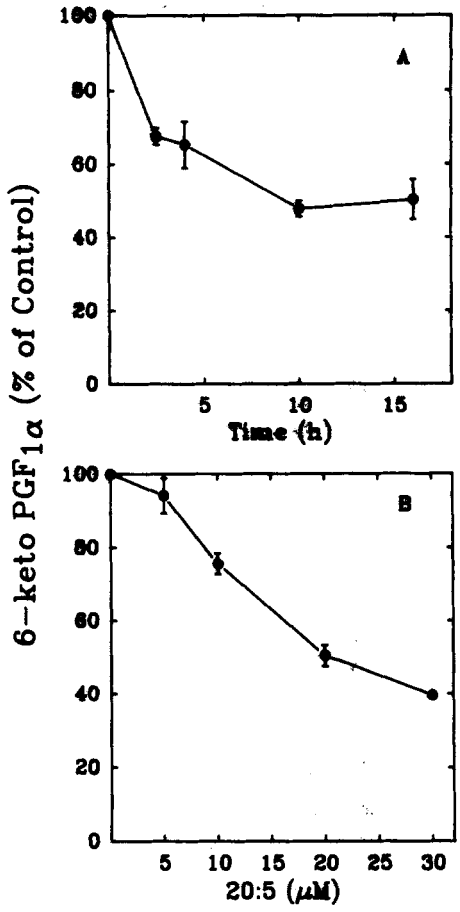

Fig. 4. Time- and concentration-dependent reduction of 6-keto $PGF_{1\alpha}$ in microvessel endothelium. (A) The confluent cultures were incubated with Lewis medium supplemented with 20 μM EPA and 1% FBS for the indicated time periods. After removing the supplemental medium, the cells were washed with serum-free medium and then incubated with fresh medium containing 2 μM ionophore A23187 for 10 min. 6-Keto $PGF_{1\alpha}$ present in the medium was measured by radioimmunoassay. (B) The cells were incubated with medium containing various amounts of EPA for 16 h. After washing, the cells were incubated with serum-free medium containing 2 μM ionophore A23187 for 10 min. 6-Keto $PGF_{1\alpha}$ present in the medium was measured by radioimmunoassay. Each data point is the mean ± SE of values obtained from three separate cultures.

Effect of other fatty acids

To determine whether the decrease in PGI_2 production by microvessel endothelial cells was specific for EPA, the effect of other fatty acids was examined. As shown in **Fig. 5A**, EPA was more effective than any of the other fatty acids tested in reducing PGI_2 formation. Linolenic [18:3(n-3)] and docosahexaenoic [22:6(n-3)] acids reduced PGI_2 formation in response to ionophore A23187, but only about 50–60% as much as EPA. PGI_2 production was not affected substantially by a prior exposure to arachidonic [20:4(n-6)], linoleic [18:2(n-6)], or palmitic (16:0) acids.

Similar results were observed regarding the effects of these fatty acids on PGE_2 production (Fig. 5B). Again, the largest decrease was produced by EPA, 18:3 and 22:6 produced moderate redutions, and the other fatty acids had little or no effect.

EPA uptake

To further evaluate the capacity of the microvessel endothelial cells to utilize EPA, the uptake of [^{3}H]EPA was compared with that of [^{3}H]arachidonic acid. As seen in **Fig. 6A**, both fatty acids were initially taken up to about the same extent, but somewhat more arachidonic acid was retained in long-term incubations. The greater retention of arachidonic acid is also illustrated in the concentration experiment, Fig. 6B, in which the time of incubation was 16 h. More arachidonic acid radioactivity than EPA remained in the cell lipids at all of the concentrations tested.

Both radioactive fatty acids were incorporated into phospholipids and neutral lipids of the microvessel endothelium. More than 95% of the neutral lipid radioactivity was contained in triglycerides in both cases. The distribution of radioactivity between phospholipids and triglycerides at the various fatty acid concentrations tested in 16 h incubation is shown in **Fig. 7**. Larger amounts of both fatty acids were

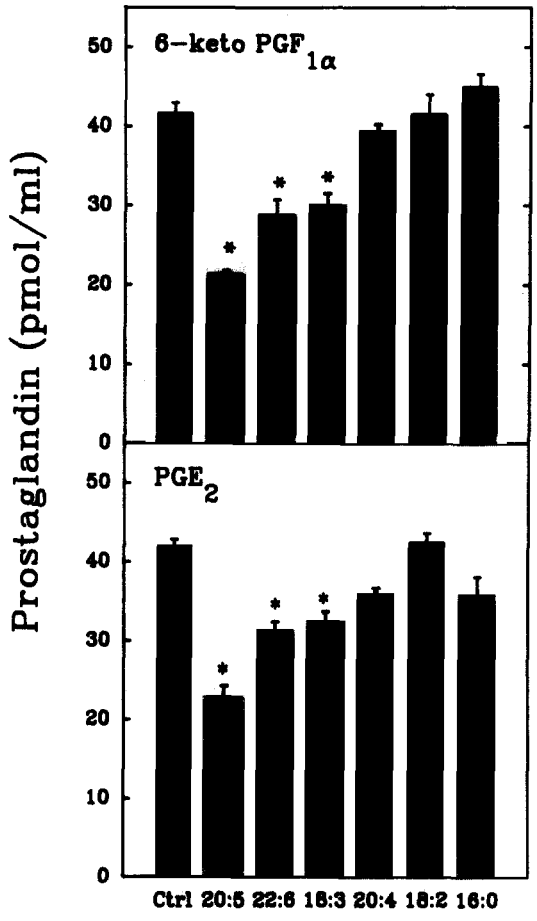

Fig. 5. Effect of different n-3 and n-6 fatty acids on 6-keto $PGF_{1\alpha}$ and PGE_2 formation. Endothelial cultures were incubated in Lewis medium containing 1% FBS and supplemented with 20 μM fatty acid. After 10 h, the medium was removed, and cells were washed and then stimulated with 2 μM ionophore A23187 for 10 min. 6-Keto $PGF_{1\alpha}$ and PGE_2 present in the medium were measured by radioimmunoassay. Each bar is the mean ± SE of values obtained from three separate cultures. The fatty acids are indicated as number of carbons:number of double bonds. The bar for control cultures incubated without supplemental fatty acids is designated as Ctrl. *, significantly less than control value ($P < 0.05$).

incorporated into phospholipids and at each concentration, 2- to 3-times more labeled arachidonic acid than EPA was recovered in this fraction. By contrast, more labeled EPA than arachidonic acid was recovered in triglycerides at each concentration tested. Furthermore, only 1.2- to 1.7-times more labeled EPA was incorporated into phospholipids than triglycerides, whereas in the case of arachidonic acid, the incorporation into phospholipids was 4- to 5-times higher.

Fig. 8 shows the distribution of these fatty acids in the microvessel endothelial phospholipids at the end of the 16-h incubation. Similar amounts of labeled EPA and arachidonic acid were contained in the choline glycerophospholipids. By contrast, much more labeled arachidonic acid was present in the other glycerophospholipid fractions.

Isolated microvessels

Isolated mouse brain microvessels produce at least five radioactive metabolites from tracer amounts of [^{3}H]arachidonic acid, as detected by HPLC separation of the medium following a 1-h incubation. This is illustrated in **Fig. 9A**. Three of the metabolites produced by the microvessel endothelial cultures were detected: PGI_2 as 6-keto-$PGF_{1\alpha}$, PGE_2, and HHT. Two additional metabolites were formed by the microvessels, $PGF_{2\alpha}$ and 12-HETE, the most abundant labeled product.

Radioactive metabolites were also produced when isolated microvessels were incubated with [^{3}H]EPA. This is shown in Fig. 9B. A small amount of radioactivity eluted in the regions expected for the PGI_3 inactivation product, Δ17-6-keto-$PGF_{1\alpha}$, $PGF_{3\alpha}$, PGE_3, and 12-hydroxyheptadecatetraenoic acid. There also were two major radioactive

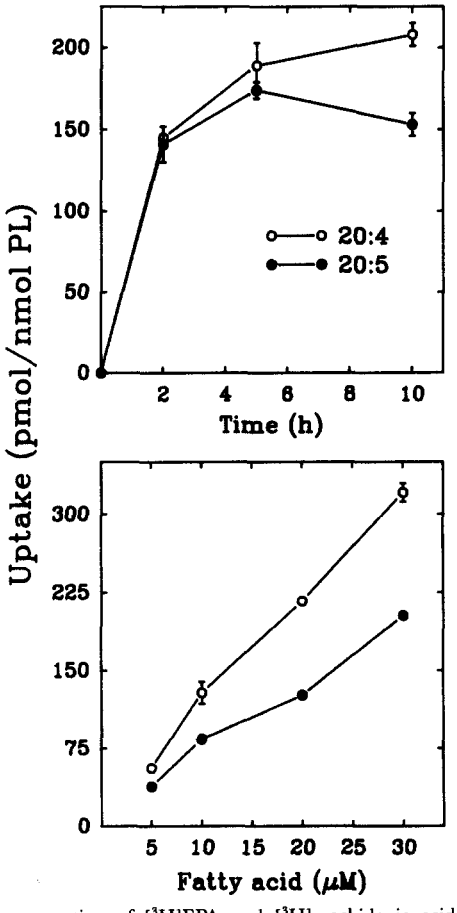

Fig. 6. Incorporation of [^{3}H]EPA and [^{3}H]arachidonic acid into cell lipids. (A) The confluent cultures were incubated with Lewis medium containing 1% FBS and 20 μM [^{3}H]EPA or [^{3}H]arachidonic acid for the indicated times. After removing the labeled medium, the cells were washed with medium containing 10 μM BSA to remove adherent fatty acids, harvested, and the lipids were extracted. Aliquots of the cell lipid extracts were assayed for radioactivity. In the concentration study (B), the cells were incubated for 16 h. Each point is the mean ± SE of values obtained from three separate cultures. Fatty acid uptake is expressed as pmol/nmol cellular phospholipid.

metabolites that have retention times expected for HEPEs.

As observed with the microvessel endothelial cultures, an initial exposure to EPA reduced the capacity of the isolated microvessels to produce PGI_2 and PGE_2. This is shown in **Table 1**. After a 2-h incubation with 20 μM EPA, there was a 38% decrease in PGI_2 formation and 67% decrease in PGE_2 formation by the microvessels in response to ionophore A23187.

DISCUSSION

Previous studies with cultured mouse brain microvascular endothelium indicated that these cells actively metabolize arachidonic acid primarily through the cyclooxygenase pathway to PGI_2 and PGE_2 (16). Prostaglandin I_2 is the major cyclooxygenase derivative produced by these cells under basal conditions or when they are provided with tracer amounts of exogenous [^{3}H]arachidonic acid. By contrast, PGE_2 becomes the predominant derivative upon incubation with 7.5 μM arachidonic acid or calcium ionophore A23187 (16). This was confirmed in the present study, which indicated that the microvessel endothelial cultures also produce small amounts of lipoxygenase products, HETEs (Fig. 1A). Under similar experimental conditions, these cells produced metabolites from EPA with the expected chromatographic properties of PGI_3, PGE_3, and hydroxylated fatty acids that may be lipoxygenase products (Fig. 1B). However, less EPA than arachidonic acid was converted to these oxygenated metabolites. While these findings demonstrate that the microvessel endothelial cells are capable of utilizing EPA as a substrate from cyclooxygenase and lipoxygenase pathways, producing eicosanoids analogous to those produced from arachidonic acid, other cultured cells such as bovine aortic endothelium (12), human umbilical vein endothelium (11, 22), and rat aortic smooth muscle (8) are reported to produce little or no metabolites from EPA. Concomitant incubation of EPA and arachidonic acid is required for human umbilical vein endothelium to produce significant quantities of PGI_3 (22), as confirmed in Fig. 2, but the simultaneous presence of arachidonic acid is not necessary for the brain microvessel endothelial cells to convert EPA to these products.

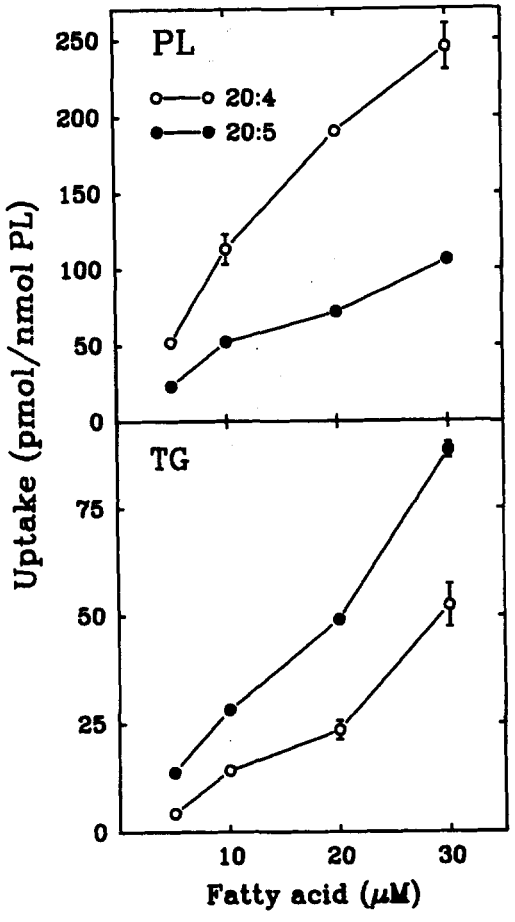

Fig. 7. Distribution of [^{3}H]arachidonic acid and [^{3}H]EPA in phospholipids (PL) and triglycerides (TG). Endothelial cells were incubated with increasing amounts of either [^{3}H]arachidonic acid or [^{3}H]EPA for 16 h. After washing, the cells were harvested, and the cell lipids were extracted and separated by TLC. Each point is the mean ± SE of values obtained from three separate cultures. Incorporation into triglycerides and phospholipids is expressed as pmol/nmol cellular phospholipid.

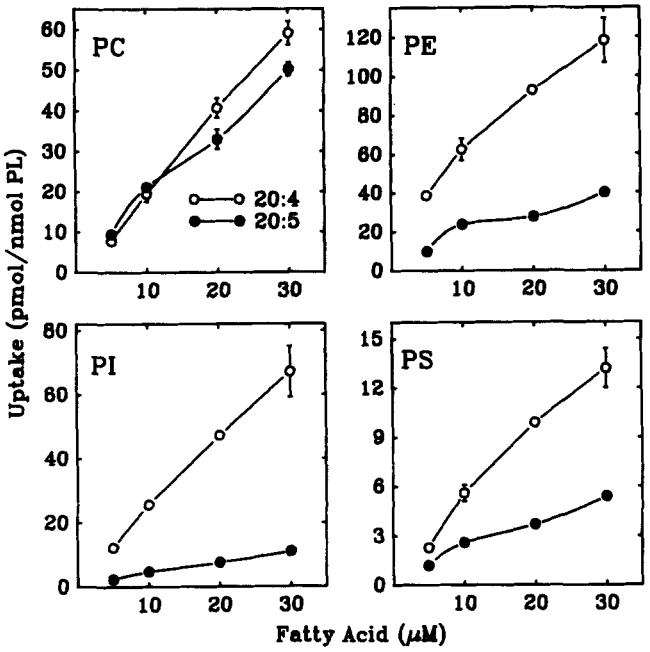

Fig. 8. [^{3}H]EPA and [^{3}H]arachidonic acid inorporation into cell phospholipids. Confluent cultures were incubated with increasing amounts of either [^{3}H]arachidonic acid or [^{3}H]EPA for 16 h. After the cells were washed and harvested, the total cell lipids were extracted and the phospholipids were separated by TLC. Each point is the mean ± SE of values obtained from three separate cultures. Incorporation into phospholipids is expressed as pmol/nmol cellular phospholipid.

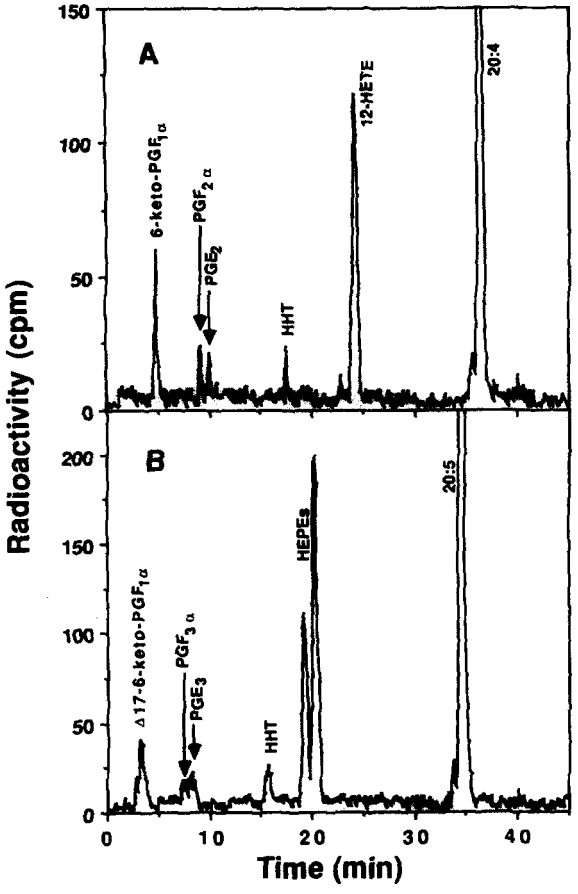

Fig. 9. Radioactive eicosanoids formed by isolated intact microvessels. The microvessel isolates were incubated with serum-free Lewis medium containing 1 μCi/ml [^{3}H]arachidonic acid (A) or [^{3}H]EPA (B) for 1 h. The medium was collected, extracted, and analyzed by HPLC. The chromatograms were obtained from microvessels pooled from four brains.

Freshly isolated murine brain microvessels also produce oxygenated derivatives of arachidonic acid and EPA (Fig. 9). As previously reported, a lipoxygenase metabolite, 12-HETE, is the major arachidonic acid derivative formed by brain microvessels (15, 23, 24), while prostaglandins I_2, E_2, and $F_{2\alpha}$ are minor products. Utilizing tracer amounts of [^{3}H]arachidonic acid, PGI_2 is the major cyclooxygenase derivative, just as it is in the cultured cells. In the present study, metabolites with the expected chromatographic properties of trienoic prostaglandins corresponding to the arachidonic acid derivatives, prostaglandins I_2, E_2, and $F_{2\alpha}$, and two compounds with chromatographic properties of HEPEs were produced from EPA by isolated brain microvessels (Fig. 9B). As was the case with arachidonic acid, the lipoxygenase derivatives were the predominant EPA metabolites. Isolated vascular tissues generally have been reported to produce little or no prostaglandins from EPA (8, 25–27), and there are no previous reports of vascular tissues producing EPA lipoxygenase metabolites. These previous studies with isolated vascular tissues have all utilized large vessels. Although large vessels are capable of producing small amounts of lipoxygenase products, most commonly 15-HETE, prostaglandins are by far the major arachidonic acid metabolites that are formed (28). This disparity in the activity of the cyclooxygenase as opposed to the lipoxygenase pathway appears to be a major difference between the arachidonic acid metabolism of large vessels and brain microvessels, a difference that also holds true for EPA metabolism.

Fisher and Weber (14) and Knapp et al. (15) demonstrated that dietary EPA was converted to PGI_3 in humans, as indicated by gas–liquid chromatography–mass spectrometry analysis of PGI_3 metabolites in urine, and suggested that endothelial cells were the major source of the PGI_3. These data, however, are inconsistent with reports of EPA metabolism in cultured large vessel endothelium indicating that these cells do not produce significant quantities of PGI_3 (11, 12). Furthermore, cultured large vessel endothelial cells produce substantial amounts of PGI_3 only when EPA and arachidonic acid are added together (22). In demonstrating that microvessel endothelium produces PGI_3, even without the concomitant addition of arachidonic acid, the present findings suggest that microvessels may be the primary source of vascular EPA metabolites.

The data of Fisher and Weber (14), Knapp et al. (15), and Croft et al. (29) also indicate that PGI_2 production is not reduced by dietary EPA. By contrast, tissue culture studies with large vessel endothelium consistently report decreased production of PGI_2 when the medium is supplemented with EPA (11, 12, 30). In an attempt to explore this inconsistency, we examined the effect of EPA on arachidonic acid metabolism in preparations of microvessel endothelium. As seen previously with large vessel endothelium, exposure of either cultured microvessel endothelium or isolated microvessels to EPA resulted in a significant reduction in both PGI_2 and PGE_2 formation (Fig. 3 and Table 1). Prostaglandin production from both exogenous and endogenous sources of arachidonic acid was reduced, but the reductions were larger when the cells were stimulated with ionophore A23187 or acetylcholine and an endogenous source of arachidonic acid

TABLE 1. Effect of exposure to EPA on prostaglandin production by isolated brain microvessels

	Amount Formed (pmol/mg protein)		
Prostaglandin	Control	EPA-Treated	*P* Value
PGI_2	25.3 ± 0.69	15.6 ± 0.47	< 0.001
PGE_2	32.6 ± 0.49	13.9 ± 0.71	< 0.001

Brain microvessels were isolated on nylon mesh and incubated in Lewis medium containing 1% FBS with or without 20 μM EPA for 2 h. After this medium was removed, the vessels were washed and incubated in serum-free Lewis medium containing 2 μM ionophore A23187 for 20 min. This medium was assayed for PGI_2 (as 6-keto $PGF_{1\alpha}$) and PGE_2 by radioimmunoassay. Each value is mean ± SE of results obtained from three separate microvessel isolates, each isolate collected from four brains.

was utilized. EPA has previously been shown to competitively block arachidonic acid metabolism at the cyclooxygenase step (31). While having a high binding affinity for cyclooxygenase, EPA is a poor substrate for the enzyme relative to arachidonic acid (13, 31). When the cells are enriched with EPA, exposure to these agonists probably produces a concomitant release of arachidonic acid and EPA from endothelial cell lipids, allowing EPA to compete with arachidonic acid for access to cyclooxygenase. Furthermore, EPA supplementation studies in vivo and in vitro indicate that the arachidonic acid content of vascular cell lipids is reduced and replaced to some extent by EPA (11, 12, 32, 33). This might further reduce the capacity of the cells to produce dienoic prostaglandins by limiting the availability of arachidonic acid.

Considerable differences were observed in the relative amounts of EPA and arachidonic acid incorporated into triglycerides and phospholipids. More EPA than arachidonic acid was incorporated into triglycerides (Fig. 7) and less EPA than arachidonic acid was incorporated into ethanolamine, inositol, and serine glycerophospholipids (Fig. 8). The substantial uptake of EPA into triglycerides is particularly intriguing, since much more arachidonic acid than EPA is incorporated into triglycerides in large vessel endothelial cells (11, 12). This suggests that triglycerides may play an important role in the processing and metabolism of EPA in brain microvessel endothelium.

Incubations with a variety of fatty acids in the present studies clearly demonstrate that only the n-3 polyunsaturated class reduces dienoic prostaglandin formation in microvascular endothelium (Fig. 5). This is another important difference from large vessel endothelium, where incubation with linoleic or arachidonic acid also reduces the capacity of the cells to subsequently produce prostaglandins (34, 35). The main prostaglandin formed by the large vessel endothelial cells is PGI_2 (34, 35), not PGE_2, one of the main prostaglandins formed by brain microvascular endothelium (Figs. 1 and 9). In this regard, exposure to linoleic or arachidonic acid does not reduce the capacity of 3T3 or MDCK cells, which also produce primarily PGE_2, to subsequently form prostaglandins (36, 37). This suggests that the difference in response of the microvessel and large vessel endothelial cells to n-6 polyunsaturated fatty acids might be related to the fact that they produce different kinds of prostaglandins.

The mechanism through which 18:3(n-3) and 22:6(n-3) reduce dienoic prostaglandin formation has not as yet been determined. Although 22:6(n-3) has been reported to directly inhibit cyclooxygenase (38), neither 18:3(n-3) nor 22:6(n-3) are substrates for this enzyme. Besides direct interaction with cyclooxygenase, elongation and desaturation of 18:3(n-3) to EPA or retroconversion of 22:6(n-3) to EPA might account for the decreases produced by these fatty acids. Studies in our laboratory indicate that cultured brain microvascular endothelium can actively convert 18:3(n-3) to EPA (Moore, S. A., E. Yoder, and A. A. Spector, unpublished data); large vessel endothelium also can convert 18:3(n-3) to EPV (11, 39). Retroconversion, however, has not yet been documented in micrivascular endothelial cells, even though other tissues, including large vessel endothelium, are capable of producing EPA in this manner (40, 41).

These studies may have important implications regarding the potential effects of EPA supplementation on cerebrovascular physiology and pathophysiology. Dietary enrichment with n-3 fatty acids in vivo modulates the fatty acyl composition of glycerolipids in cerebral microvessels (32, 42, 43). Brown, Marshall, and Johnston (32) demonstrated that such dietary modification raises the EPA content of choline and ethanolamine phospholipids in brain microvessels, while reducing brain microvessel PGI_2 production. These results are consistent with the present findings, even though Brown et al. (32) utilized long-term dietary modification in vivo and we utilized a short-term incubation of the isolated microvessels or the cultured endothelium with media containing supplemental EPA. While the potential physiological effects of EPA supplementation are uncertain at this juncture, one in vivo study suggests that dietary EPA has a beneficial effect on the cerebral vasculature following brain ischemia (5). Gerbils fed a menhaden oil diet through two generations had no decrease in cerebral blood flow and no cerebral edema in response to carotid artery ligation followed by reperfusion. By contrast, there was a 25% drop in cerebral blood flow accompanied by the formation of cerebral edema in gerbils fed a standard diet (5). Dienoic prostaglandin production in whole brain homogenates was not altered by the fish oil diet, but trienoic prostaglandin and pentaenoic lipoxygenase product formation were not assessed. If eicosanoids are produced from EPA in vivo to the extent observed in the present study, they may underlie some of the protective effects noted by Black et al. (5) through anti-platelet activity or direct vasoactive actions.

This work was supported by Research Grant HL-39308 from the National Heart, Lung, and Blood Institute, National Institutes of Health, and Grants NS-01096 and NS-24621 from the National Institute of Neurological Disorders and Stroke. The authors would like to thank Jacqueline Kjaer for typing and Joel Carl for preparation of illustrations.

Manuscript received 3 April 1989 and in revised form 26 May 1989.

REFERENCES

1. Dyerberg, J., and H. O. Bang. 1979. Haemostatic function and platelet polyunsaturated fatty acids in Eskimos. *Lancet.* **ii:** 433-435.
2. Dyerberg, J., H. O. Bang, E. Stoffersen, S. Moncada, and J. R. Vane. 1978. Eicosapentaenoic acid and prevention of thrombosis and atherosclerosis. *Lancet.* **ii:** 117-119.
3. Kromhout, D., E. B. Bosschieter, and C. D. Coulander. 1985.

The inverse relation between fish consumption and 20-year mortality from coronary heart disease. *N. Engl. J. Med.* **312:** 1205–1209.
4. Lee, T. H., R. L. Hoover, J. D. Williams, R. I. Sperling, J. Ravalese, B. W. Spur, D. R. Robinson, E. J. Corey, R. A. Lewis, and K. F. Austen. 1985. Effects of dietary enrichment with eicosapentaenoic and docosahexaenoic acids on in vitro neutrophil and monocyte leukotriene generation and neutrophil function. *N. Engl. J. Med.* **312:** 1217–1224.
5. Black, K. L., J. T. Hoff, N. S. Radin, and G. D. Deshmukh. 1984. Eicosapentaenoic acid: effect on brain prostaglandins, cerebral blood flow and edema in ischemic gerbils. *Stroke.* **15:** 65–69.
6. Siess, W., P. Roth, B. Scherer, I. Kurzmann, B. Böhlig, and P. C. Weber. 1980. Platelet-membrane fatty acids, platelet aggregation, and thromboxane formation during a mackerel diet. *Lancet.* **i:** 441–444.
7. Straser, T., S. Fisher, and P. C. Weber. 1985. Leukotriene B_5 is formed in human neutrophils after dietary supplementation with eicosapentaenoic acid. *Proc. Natl. Acad. Sci. USA.* **821:** 1540–1543.
8. Morita, I., Y. Saito, W. C. Chang, and S. Murota. 1983. Effects of purified eicosapentaenoic acid on arachidonic acid metabolism in cultured murine aortic smooth muscle cells, vessel walls and platelets. *Lipids.* **18:** 42–49.
9. Lokesh, B. R., J. M. Black, J. B. German, and J. E. Kinsella. 1988. Docosahexaenoic acid and other dietary polyunsaturated fatty acid suppress leukotriene synthesis by mouse peritoneal macrophages. *Lipids.* **23:** 968–978.
10. Takahata, K., and M. Yamanaka. 1987. PGI_3 production from eicosapentaenoic acid in 3T3 fibroblast cells and cultured bovine pulmonary artery endothelial cells. *Thromb. Res.* **45:** 581–589.
11. Spector, A. A., T. L. Kaduce, P. H. Figard, K. C. Norton, J. C. Hoak, and R. L. Czervionke. 1983. Eicosapentaenoic acid and prostacyclin production by cultured human endothelial cells. *J. Lipid Res.* **24:** 1595–1604.
12. Hadjiagapiou, C., T. L. Kaduce, and A. A. Spector. 1986. Eicosapentaenoic acid utilization by bovine aortic endothelial cells: effects on prostacyclin production. *Biochim. Biophys. Acta.* **875:** 369–381.
13. Lands, W. E. M., and M. J. Byrnes. 1981. The influence of ambient peroxides on the conversion of 5,8,11,14,17-eicosapentaenoic acid to prostaglandins. *Prog. Lipid Res.* **20:** 287–290.
14. Fisher, S., and P. C. Weber. 1984. Prostaglandin I_3 is formed in vivo in man after dietary eicosapentaenoic acid. *Nature.* **307:** 165–168.
15. Knapp, H. H., I. A. G. Reilly, P. Alessandrini, and G. A. Fitzgerald. 1986. In vivo indexes of platelet and vascular function during fish-oil administration in patients with atherosclerosis. *New Engl. J. Med.* **314:** 937–942.
16. Moore, S. A., A. A. Spector, and M. N. Hart. 1988. Eicosanoid metabolism in cerebromicrovascular endothelium. *Am. J. Physiol.* **254:** C37–C44.
17. Sahagan, G., S. A. Moore, Z. Fabry, R. L. Schelper, and M. N. Hart. 1989. Purification of murine endothelial cell cultures by flow cytometry using fluorescein-labeled *Griffonia simplicifolia* agglutinin. *Am. J. Pathol.* **134:** 1227–1232.
18. Lessard, J., C. Adelbrook, D. Carlton, D. Lund, and S. Dingle. 1984. Monoclonal antibodies to arterial actins. In International Cell Biology. F. Sero and Y. Okada, editors. Japan Society of Cell Biology, Kyoto. 515.
19. Folch, J. M., M. Lees, and G. H. Sloane Stanley. 1954. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
20. Eling, T., B. Tainer, A. Ally, and R. Warnock. 1982. Separation of arachidonic acid metabolites by high-pressure liquid chromatography. *Methods Enzymol.* **86:** 511–517.
21. Chalvardjian, A., and E. Rudnicki. 1970. Determination of lipid phosphorus in the nanomolar range. *Anal. Biochem.* **36:** 225–226.
22. Bordet, J. C., M. Guichardant, and M. Lagarde. 1986. Arachidonic acid strongly stimulates prostaglandin I_3 (PGI_3) production from eicosapentaenoic acid in human endothelial cells. *Biochem. Biophys. Res. Commun.* **135:** 403–410.
23. Hambrecht, G. S., S. A. Adesuyi, S. Holt, and E. F. Ellis. 1987. Brain 12-HETE formation in different species, brain regions, and in brain microvessels. *Neurochem. Res.* **12:** 1029–1033.
24. Moore, S. A., P. H. Figard, A. A. Spector, and M. N. Hart. 1989. Brain microvessels produce 12-hydroxyeicosatetraenoic acid. *J. Neurochem.* **53:** 376–382.
25. Hornstra, G., E. Christ-Hazelhof, E. Haddeman, F. ten Hoor, and D. H. Negteren. 1981. Fish oil feeding lowers thromboxane and prostacyclin production by rat platelets and aorta and does not result in the formation of prostaglandin I_3. *Prostaglandins* **21:** 727–738.
26. Dyerberg, J., K. A. Jorgensen, and T. Arnfred. 1981. Human umbilical blood vessel converts all *cis*-5,8,11,14,17 eicosapentaenoic acid to prostaglandin I_3. *Prostaglandins* **22:** 857–862.
27. Kristensen, S. D., T. Arnfred, and J. Dyerberg. 1984. Eicosapentaenoic acid potentiates the production of prostacyclin-like material in the arachidonic acid-perfused human umbilical vein. *Thromb. Res.* **36:** 305–314.
28. Powell, W. S., and C. D. Funk. 1987. Metabolism of arachidonic acid and other polyunsaturated fatty acids by blood vessels. *Prog. Lipid Res.* **26:** 183–210.
29. Croft, K. D., L. J. Bellin, F. M. Legge, and R. Vandongen. 1987. Effects of diets enriched ineicosapentaenoic or docosahexaenoic acids on prostanoid metabolism in the rat. *Lipids.* **22:** 647–650.
30. Brox, J. H., and A. Nordoy. 1983. The effect of polyunsaturated fatty acids on endothelial cells and their production of prostacyclin, thromboxane and platelet inhibitory activity. *Thromb. Haemostasis.* **50:** 762–767.
31. Needleman, P., A. Raz, M. S. Minkes, J. A. Ferrendelli, and H. Sprecher. 1979. Triene prostaglandins: prostacyclin and thromboxane biosynthesis and unique biological properties. *Proc. Natl. Acad. Sci. USA.* **76:** 944–948.
32. Brown, M. L., L. A. Marshall, and P. V. Johnston. 1984. Alterations in cerebral and microvascular prostaglandin synthesis by manipulation of dietary essential fatty acids. *J. Neurochem.* **43:** 1392–1400.
33. Takahashi, R., B. A. Nassar, Y-S. Huang, M. E. Begin, and D. F. Horrobin. 1987. Effect of different ratios of dietary n–6 and n–3 fatty acids on fatty acid composition, prostaglandin formation and platelet aggregation in the rat. *Thromb. Res.* **47:** 135–146.
34. Spector, A. A., J. C. Hoak, G. L. Fry, G. M. Denning, L. L. Stoll, and J. B. Smith. 1980. Effect of fatty acid modification on prostacyclin production by cultured human endothelial cells. *J. Clin. Invest.* **65:** 1003–1012.
35. Spector, A. A., T.L. Kaduce, J. C. Hoak, and R. L. Czervionke. 1983. Arachidonic acid availability and prostacyclin production by cultured human endothelial cells. *Arteriosclerosis.* **3:** 323–331.
36. Denning, G. M., P. H. Figard, and A. A. Spector. 1982. Effect of fatty acid modification on prostaglandin production by cultured 3T3 cells. *J. Lipid Res.* **23:** 584–596.
37. Lewis, M. L., T. L. Kaduce, and A. A. Spector. 1981. Effect

of essential polyunsaturated fatty acid modifications on prostaglandin production by MDCK canine kidney cells. *Prostaglandins.* **22:** 747–760.

38. Croset, M., M. Guichardant, and M. Lagarde. 1988. Different metabolic behavior of long-chain n–3 polyunsaturated fatty acids in human platelets. *Biochim. Biophys. Acta.* **961:** 262–269.
39. Rosenthal, M. D., and C. Whitehurst. 1983. Fatty acyl Δ^6 desaturation activity of cultured human endothelial cells. Modulation by fetal bovine serum. *Biochim. Biophys. Acta.* **750:** 490–496.
40. Fischer, S., A. Vischer, V. Preac-Mursic, and P. C. Weber. 1987. Dietary docosahexaenoic acid is retroconverted in man to eicosapentaenoic acid, which can be quickly transformed to prostaglandin I_3. *Prostaglandins.* **34:** 367–375.
41. Hadjiagapiou, C., and A. A. Spector. 1987. Docosahexaenoic acid metabolism and effect on prostacyclin production in endothelial cells. *Arch. Biochem. Biophys.* **253:** 1–12.
42. Selivanchick, D. P., and B. I. Roots. 1979. Isolated brain capillary endothelia: influence of various levels of essential fatty acids on the acyl composition of glycerophosphatides. *Lipids.* **14:** 66–69.
43. Matheson, D. F., R. Oei, and B. I. Roots. 1980. Influence of diet on the acyl composition of phospholipids in endothelial cells and mitochondria of rat brain. *Neurochem. Res.* **5:** 43–50.