Eicosapentaenoic acid and prostacyclin production by cultured human endothelial cells

Arthur A. Spector,[1] Terry L. Kaduce, Paul H. Figard, Kurt C. Norton, John C. Hoak, and Robert L. Czervionke

Departments of Biochemistry and Internal Medicine, University of Iowa, Iowa City, IA 52242

Abstract Human umbilical vein endothelial cells incorporate eicosapentaenoic acid (EPA) when this fatty acid is present in the culture medium. From 30 to 70% of the uptake remains as EPA, and much of the remainder is elongated to docosapentaenoic acid. All of the cellular glycerophospholipids become enriched with EPA and docosapentaenoic acid, with the largest increase in EPA occurring in the choline glycerophospholipids. When this fraction is enriched with EPA, it exhibits a large decrease in arachidonic acid content. Cultures exposed to tracer amounts of [1-^{14}C]linolenic acid in 5% fetal bovine serum convert as much as 17% of the radioactivity to EPA. The conversion is reduced, however, in the presence of either 20% fetal bovine serum or 50 μM linolenic acid. Like arachidonic acid, some newly incorporated EPA was released from the endothelial cells when the cultures were exposed to thrombin. However, as compared with arachidonic acid, only very small amounts of EPA were converted to prostaglandins. Cultures enriched with EPA exhibited a 50 to 90% reduction in capacity to release prostacyclin (PGI_2) when subsequently stimulated with thrombin, calcium ionophore A23187, or arachidonic acid. The degree of inhibition was dependent on the time of exposure to EPA and the EPA concentration, and it was not prevented by adding a reversible cyclooxygenase inhibitor, ibuprofen, during EPA supplementation. EPA appears to decrease the capacity of the endothelial cells to produce PGI_2 in two ways: by reducing the arachidonic acid content of the cell phospholipid precursor pools and by acting as an inhibitor of prostaglandin production. These findings suggest that regimens designed to reduce platelet aggregation and thrombosis by EPA enrichment may also reduce the capacity of the endothelium to produce PGI_2.—**Spector, A. A., T. L. Kaduce, P. H. Figard, K. C. Norton, J. C. Hoak, and R. L. Czervionke.** Eicosapentaenoic acid and prostacyclin production by cultured human endothelial cells. *J. Lipid Res.* 1983. **24:** 1595–1604.

Supplementary key words prostaglandins • linolenic acid • arachidonic acid • phospholipids • docosapentaenoic acid

The n-3 class of polyunsaturated fatty acids, particularly eicosapentaenoic acid (EPA), may be useful in preventing the thrombotic complications of atherosclerotic cardiovascular disease (1). This is based on the observation that the Greenland Eskimos, whose diet is rich in n-3 polyunsaturates, have a prolonged bleeding time and a very low incidence of cardiovascular deaths (2). Platelet membranes become enriched with EPA when the diet is rich in EPA, and this reduces thromboxane production and platelet aggregation (3). Platelets also become enriched with EPA when they are incubated with this fatty acid in vitro, and some of the newly incorporated EPA is released when the platelets are exposed to thrombin (4, 5). EPA reduces platelet aggregation by decreasing the conversion of arachidonic acid to thromboxane A_2 and by itself being converted to prostaglandin D_3, an anti-aggregatory substance (6, 7).

While there is general agreement regarding the effects of EPA on platelets, the available information regarding vascular tissue is less certain. For example, pieces of rat aorta have been observed to convert EPA to an anti-aggregatory material, believed to be prostaglandin I_3 (8, 9). Likewise, human umbilical blood vessels are reported to convert EPA to prostaglandin I_3, although at only 50% of the rate at which they convert arachidonic acid to PGI_2 (10, 11). EPA did not inhibit the conversion of arachidonic acid to PGI_2 by the umbilical blood vessels (10), and it appeared to actually increase PGI_2 production in rat aorta (12). Conversely, others have observed that rat aorta does not convert EPA to prostaglandin I_3 and that enrichment with EPA reduces PGI_2 production by 35% (13). Likewise, cultured murine smooth muscle cells are reported not to convert EPA to prostaglandin I_3 and to produce less PGI_2 from arachidonic acid after incorporation of EPA (14).

Some of the inconsistency in the reported effects of EPA on PGI_2 and prostaglandin I_3 production may be due to the fact that many different types of preparations have been studied and, in the cases where blood vessels were tested, the different cell types present in the preparation. An important source of vascular PGI_2 is the endothelium (15), and cultured endothelial cell preparations that produce PGI_2 are now available (16, 17). In an attempt to define the effects of EPA on vascular tissue

Abbreviations: EPA, eicosapentaenoic acid; PGI_2, prostaglandin I_2 or prostacyclin; 6-keto-$PGF_{1\alpha}$, the 6-keto-derivative of prostaglandin $F_{1\alpha}$.

[1] Please address reprint requests to Dr. Spector, Department of Biochemistry.

better, we have investigated its utilization and influence on prostaglandin production by human umbilical vein endothelial cells.

MATERIALS AND METHODS

Reagents

Fatty acids were obtained from Nu-Chek Prep (Elysian, MN). Bovine albumin (Fraction V, fatty acid-free) was purchased from Miles Laboratories, Inc. (Elkhart, IN). The calcium ionophore A23187 was supplied by Calbiochem-Behring (La Jolla, CA). [1-^{14}C]Arachidonic acid and [1-^{14}C]eicosapentaenoic acid (40–60 mCi/mmol) were purchased from New England Nuclear (Boston, MA).

Endothelial cell culture

Endothelial cells were obtained from human umbilical veins (18), and primary cultures were prepared according to a slight modification of the method of Jaffe et al. (19) as previously described (20, 21). Briefly, the cells were suspended in a modified Medium 199 containing 20% heat-inactivated fetal bovine serum, counted with a hemocytometer, and seeded in 25-cm^2 flasks at a concentration of 2.25×10^6 cells per flask. After incubation for 24 hr at 37°C in an atmosphere of 5% CO_2, this medium was replaced with 3 ml of Medium 199 containing 25 mM Hepes plus 20% fetal bovine serum, and the confluent cultures were continued in the 5% CO_2 atmosphere. Most of the experiments with fatty acid were done with a culture medium containing Medium 199, 25 μM Hepes, 2.5% fetal bovine serum, and 90 μM bovine albumin. These media were enriched with fatty acids by adding a warm solution of the sodium salt (20). The pH was adjusted to 7.4 at 37°C, and the media were sterilized by filtration through 0.22-μm filters.

Incubation and lipid analysis

After the maintenance medium was removed, 2 ml of the fatty acid-supplemented medium was added and the incubations were carried out at 37°C with a 5% CO_2 atmosphere. The incubations were terminated by removing the medium, washing the monolayer with 2 ml of ice-cold Dulbecco's phosphate-buffered saline solution containing 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4 (pH 7.4), and 50 μM bovine albumin, followed by a second washing with this solution containing no albumin. After harvesting by scraping, the cells were suspended in 0.5 ml of fresh, cold buffer. Previous studies with radioactive fatty acids indicated that this scraping procedure did not cause an excessive hydrolysis of fatty acids from phospholipids, as compared with other currently available methods for cell harvesting (22). A portion of the cell suspension was removed for determination of the protein content (23). The remainder of the suspension was extracted with 20 volumes of chloroform–methanol 2:1 (v/v) to isolate the cell lipids (24). Following separation and isolation of the chloroform phase, the solvent was evaporated under N_2, and the lipid residue was dissolved in a measured volume of chloroform. A portion of this solution was taken for measurement of radioactivity.

Separation of phospholipids and neutral lipids was obtained by thin-layer chromatography on silica gel G with a solvent system containing hexane–diethyl ether–methanol–acetic acid 170:40:4:4. Standards obtained from Nu-Chek Prep were added to each chromatogram, and the lipids were visualized by exposure to I_2 vapor. After sublimation of the I_2, the outlined segments of silica gel were scraped into liquid scintillation counting vials.

Phospholipids were separated by high-performance liquid chromatography on 5 μM Ultrasphere-Si using a solvent system consisting of acetonitrile–methanol–sulfuric acid 100:3:0.05. Radioactivity was measured in aliquots of the collected eluate, and additional aliquots were transesterified with 1 ml of 14% BF_3 in methanol at 100°C for 2 hr (25). The fatty acid methyl esters were separated by gas–liquid chromatography using a 2 mm × 1.9-m glass column packed with 10% SP2330 on 100/120 mesh Chromosorb W-AW (Supelco, Bellefonte, PA) (26). A Hewlett-Packard model 5700 gas chromatograph with a flame ionization detector was used. N_2 served as the carrier gas at a flow rate of 25 ml/min, and the oven temperature was programmed from 176° to 220°C. Peak areas were determined with a Hewlett-Packard model 3380S integrator-recorder, and the areas are reported as weight percentages. In several experiments radioactivity present in the effluent stream was diverted by the stream splitter through a heated collecting port and trapped in a 5-cm length of Teflon tubing immersed in liquid scintillation solution (22).

Radioactivity was measured in a liquid scintillation spectrometer using counting vials containing 4 ml of Budget-Solve scintillation solution (Research Products International, Elk Grove Village, IL). Quenching was monitored with a ^{226}Ra external standard.

6-Keto-PGF$_{1\alpha}$ assay

After the experimental medium was removed, the monolayer of intact, confluent endothelial cells was washed with Dulbecco's phosphate-buffered saline at 37°C. The cultures then were washed again with this solution containing 50 μM bovine albumin. This washing was used in an attempt to remove any free fatty acid that remained attached to the cells, a precaution that was considered especially important when the incubation me-

dium contained supplemental fatty acids. The monolayers then were incubated for 5 min at 37°C with 1 ml of 50 μM bovine albumin in Dulbecco's saline buffer as a control, or this solution containing either 1 U/ml of bovine thrombin, 10 μM calcium ionophore A23187, or arachidonic acid bound to bovine serum albumin. PGI_2 contained in the supernatant solution was measured by a radioimmunoassay for 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), a stable catabolic product of PGI_2 (20, 27).

The radioimmunoassay was performed using an antiserum against 6-keto-$PGF_{1\alpha}$. One-hundred μl of either standard or samples was mixed with 100 μl of [^{3}H]6-keto-$PGF_{1\alpha}$ (New England Nuclear, Boston, MA) and 10 μl of antiserum at a dilution that bound 50% of the radioactivity in the absence of standard. After 1 hr of incubation at 37°C, 50 μl of IgGsorb (The Enzyme Center Inc., Tufts University School of Medicine, Boston, MA) was added, and the radioactivity contained in 100 μl of the supernatant solution was measured in a liquid scintillation spectrometer. Assay detection limits were 0.3 pmol of 6-keto-$PGF_{1\alpha}$, and 50% inhibition was obtained with 3.3 pmol of 6-keto-$PGF_{1\alpha}$. This assay has negligibly small amounts of cross-reactivity with prostaglandins other than 6-keto-$PGF_{1\alpha}$.

Formation of radioactive prostaglandins

Endothelial cell monolayers were incubated for 5 min at 37°C with 2.5 ml of medium containing 6 nmol of either [1-^{14}C]arachidonic acid or [1-^{14}C]eicosapentaenoic acid, 20 nmol of unlabeled fatty acid, and 0.5 μM bovine albumin in Dulbecco's saline buffer adjusted to pH 7.4. Corresponding control incubations were done in culture dishes without cells. After incubation, the medium was removed, acidified to pH 3.5 with 1 M citric acid, and extracted twice with two volumes of ethyl acetate after saturation with NaCl (28). Aliquots of the ethyl acetate were chromatographed along with standards using the organic phase of a mixture containing ethyl acetate–2,2,4-trimethylpentane–water–acetic acid 110:50:100:20 (16). After staining with I_2 vapor and sublimation, the radioactivity contained in the outlined segments of silica gel was measured in a liquid scintillation spectrometer. The background radioactivity in the empty dish control incubations was subtracted from the quantities recovered from the endothelial cell incubations.

Release of radioactive fatty acids

Human endothelial cells were incubated for 30 min at 37°C with 1 ml of either [1-^{14}C]arachidonic acid or [1-^{14}C]eicosapentaenoic acid (~10^6 dpm). The medium was removed, and the cells were washed with warm Dulbecco's phosphate-buffered saline followed by 50 μM bovine albumin in this solution. One set of dishes was harvested and extracted with chloroform–methanol 2:1 to measure the incorporated radioactivity. Another set was incubated 5 min at 37°C with 1 ml of 50 μM bovine albumin in Dulbecco's saline buffer, and a third set was incubated 5 min at 37°C with 1 ml of thrombin (1 U/ml) in 50 μM bovine albumin in Dulbecco's saline. The media were removed and the radioactivity present as free fatty acid was determined following separation by thin-layer chromatography.

RESULTS

EPA formation and uptake

Relatively large quantities of [1-^{14}C]linolenic acid were taken up by the human umbilical vein endothelial cultures. When the medium contained 5% fetal bovine serum, 17% of the linolenic acid radioactivity recovered in the cells after a 48-hr incubation was converted to 20:5,[2] and an additional 17% was converted to 22:5. This is shown in **Table 1.** When the content of fetal bovine serum in the culture medium was raised to 20%, the total uptake of linolenic acid radioactivity decreased, and a lesser percentage of the radioactivity was converted to 20:5 and 22:5. There was no change, however, in the amount of [1-^{14}C]linolenic acid radioactivity elongated to 20:3. Much more of the radioactive uptake remained as 18:3 when the cultures were incubated with 50 μM [1-^{14}C]linolenic acid added to 90 μM bovine serum albumin. Under these conditions only 3.6% of the radioactivity was converted to 20:5 and 1.4% to 22.5. These results, which indicate that the conversion of linolenic acid to more unsaturated products is reduced when the serum or fatty acid concentration is raised, are consistent with the findings of Rosenthal and Whitehurst (29).

The endothelial cell cultures took up about 50% of the available [1-^{14}C]EPA during a 48-hr incubation (Table 1). As compared with the linolenic acid results, there was a much smaller reduction in the amount of EPA taken up when either the fetal bovine serum in the culture medium was raised from 5 to 20% or the EPA concentration was raised from 3 to 50 μM. With 3 μM EPA, more radioactivity was recovered as 22:5 than 20:5, but this was reversed when the EPA concentration was raised to 50 μM. In every case some EPA radioactivity was converted to 22:6.

Fig. 1 shows the time dependence of [1-^{14}C]EPA incorporation into the endothelial cell phospholipids. Incorporation continued throughout the 24-hr incubation period, but the rate was somewhat slower between 8 hr

[2] The fatty acids are abbreviated as number of carbon atoms: number of double bonds. Thus, 20:5 contains 20 carbon atoms and 5 double bonds.

TABLE 1. Elongation and desaturation of linolenic and eicosapentaenoic acids

	Radioactivity Incorporated[a]					
	[1-^{14}C]Linolenic Acid			[1-^{14}C]Eicosapentaenoic Acid		
	3 μM[b]		50 μM	3 μM		50 μM
Chromatography Fraction	5%[c]	20%	2.5%[d]	5%	20%	2.5%[d]
	%					
18:3	36.5 ± 1.3	36.5 ± 5.2	79.0 ± 2.6			
20:3	19.0 ± 1.6	21.3 ± 0.1	7.1 ± 0.7			
20:5	17.4 ± 1.6	13.6 ± 1.0	3.6 ± 0.7	39.1 ± 0.7	33.1 ± 0.9	62.0 ± 2.3
22:5	16.6 ± 2.5	12.7 ± 1.2	1.4 ± 0.1	43.4 ± 2.9	41.1 ± 5.8	2.2 ± 0.9
22:6	5.3 ± 0.8	7.6 ± 1.2	1.4 ± 0.2	17.5 ± 3.5	24.8 ± 4.3	10.0 ± 2.0
Percentage of radioactivity taken up[e]	45 ± 3	23 ± 2	19 ± 1	55 ± 4	50 ± 1	46 ± 0.4

[a] The incubation was for 48 hr. Each value is the mean ± SE values obtained from three separate cultures. The values for the individual fatty acids do not add up to 100% because small amounts of radioactivity were recovered in other fractions.
[b] Concentration of the radioactive fatty acid substrate.
[c] Percentage of fetal bovine serum present in the culture medium.
[d] In addition to 2.5% fetal bovine serum, these media also contained 90 μM bovine serum albumin.
[e] The media contained 427,300 dpm [1-^{14}C]linolenic acid or 451,000 dpm [1-^{14}C]EPA.

and 24 hr. The amounts of [1-^{14}C]EPA incorporated were very similar to those obtained in a corresponding set of cultures incubated with [1-^{14}C]arachidonic acid. Of the

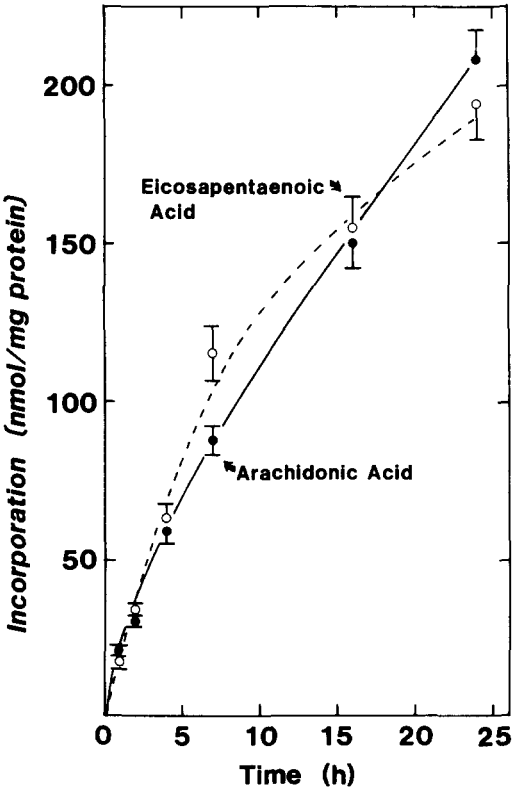

Fig. 1. Incorporation of radioactive EPA and arachidonic acid into endothelial cell phospholipids. The incubation media contained 100 μM fatty acid, 90 μM bovine serum albumin, and 2.5% fetal bovine serum. One set of cultures was incubated with [1-^{14}C]EPA, the other with [1-^{14}C]arachidonic acid. Each value is the mean ± SE of results from three separate cultures.

EPA radioactivity incorporated into phospholipids after 24 hr of incubation, 71% was recovered in choline glycerophospholipids, 16% in ethanolamine glycerophospholipids, 4% in serine glycerophospholipids, 3% in inositol glycerophospholipids, and 6% in unidentified phospholipid components.

Changes in phospholipid fatty acid composition

When endothelial cell cultures were incubated for 14 hr with 100 μM EPA, 20:5 accumulated in the major cellular phospholipid fractions. This is shown in **Table 2.** All of the glycerophospholipids except the serine fraction contained a higher percentage of polyunsaturated fatty acids when the medium was supplemented with EPA. As compared with the unsupplemented control cultures, all of the glycerophospholipid fractions of the cells exposed to supplemental EPA also were enriched in 22:5 but not 22:6. The choline glycerophospholipids exhibited the largest increase in 20:5, whereas equally large increases in 22:5 occurred in both the choline and ethanolamine glycerophospholipids. These two fractions also exhibited the largest decreases in 20:4 content when the cultures were supplemented with EPA.

Release of EPA

The retention of cellular EPA was assessed from isotopic studies. Endothelial cell cultures were labeled with [1-^{14}C]EPA for 30 min and, for comparison, corresponding cultures were labeled with [1-^{14}C]arachidonic acid. After washing, the cultures were incubated for 5 min with 1 ml of either 50 μM bovine albumin or albumin plus 1 U of bovine thrombin. As seen in **Table 3,** some of the newly incorporated radioactivity was released when the medium contained albumin alone, but considerably

TABLE 2. Fatty acid composition of the endothelial cell glycerophospholipids[a]

Fatty Acid Fraction	Composition[b]							
	Choline Glycerophospholipids		Ethanolamine Glycerophospholipids		Inositol Glycerophospholipids		Serine Glycerophospholipids	
	Control[c]	EPA	Control	EPA	Control	EPA	Control	EPA
	%							
Classes[d]								
Saturated	39 ± 2	32 ± 3	42 ± 2	37 ± 1	60 ± 3	45 ± 7	54 ± 2	50 ± 2
Monoenoic	25 ± 2	24 ± 1	21 ± 1	18 ± 1	13 ± 2	17 ± 1	16 ± 3	19 ± 1
Polyenoic	31 ± 3	41 ± 2	35 ± 2	42 ± 2	24 ± 2	32 ± 4	27 ± 3	27 ± 2
Acids								
20:4	14.5 ± 1.8	8.9 ± 1.5	12.3 ± 0.5	9.7 ± 0.6	11.1 ± 2.5	10.3 ± 1.7	5.9 ± 0.6	4.8 ± 0.6
22:4	3.5 ± 0.1	4.2 ± 0.1	7.7 ± 1.7	5.9 ± 0.1	6.0 ± 1.3	5.1 ± 0.7	7.3 ± 2.8	5.4 ± 0.9
20:5	0.7 ± 0.1	9.0 ± 2.0	1.0 ± 0.5	6.5 ± 0.7	1.1 ± 0.7	6.8 ± 0.4	2.1 ± 0.6	3.5 ± 0.5
22:5	2.4 ± 0.4	9.2 ± 2.2	3.0 ± 0.2	10.0 ± 0.5	0.8 ± 0.6	4.6 ± 0.3	2.3 ± 0.1	6.0 ± 0.5
22:6	3.0 ± 0.6	2.8 ± 1.0	3.5 ± 0.2	3.5 ± 0.1	nd[e]	nd	1.6 ± 0.1	1.6 ± 0.2

[a] Confluent cultures were incubated for 24 hr in a medium containing 90 μM bovine albumin and 2.5% fetal bovine serum.
[b] Each value is the mean ± SE of results from three separate cultures.
[c] The control cultures did not contain supplemental fatty acid. The other cultures were supplemented with 100 μM EPA.
[d] These values do not add up to 100% because some of the minor fatty acid components were not identified.
[e] Not detected.

more release occurred when the medium also contained thrombin. The release stimulated by thrombin was somewhat larger in the cells labeled with EPA than in those labeled with arachidonic acid.

PGI_2 production

The capacity of the endothelial cells to produce PGI_2 decreased when the cultures were enriched with EPA. As shown in **Fig. 2,** less PGI_2 release, measured as 6-keto $PGF_{1\alpha}$, occurred when the cultures were stimulated with three different substances that cause prostaglandin production. Arachidonic acid produced the largest PGI_2 release from the unsupplemented cultures; this was 40% greater than with the calcium ionophore A23187 and ten times greater than with thrombin. In each case, previous exposure of the cells to media supplemented with EPA reduced the PGI_2 output. In general, the extent of the reduction was dependent on the EPA concentration of the medium used initially to enrich the cultures. As compared with the corresponding unsupplemented control, the maximum reduction in PGI_2 output resulting from EPA supplementation was 50% with arachidonic acid, 75% with calcium ionophore A23187, and 95% with thrombin.

The degree of inhibition of PGI_2 production with extracellular arachidonic acid was dependent on the con-

TABLE 3. Thrombin-stimulated fatty acid release

Parameter	Radioactivity[a]			
	[1-^{14}C]EPA		[1-^{14}C]Arachidonic Acid	
	Amount	Percentage	Amount	Percentage
	dpm	%	*dpm*	%
Incorporation[b]	119,100 ± 5400		110,400 ± 13,800	
Release to albumin[c]	10,000 ± 500	8.5	6,230 ± 60	5.6
Release with thrombin[d]	23,400 ± 400	19.6	14,200 ± 200	12.8
Thrombin-stimulated release[e]	13,400	11.3	7,970	7.2

[a] Values are mean ± SE of results obtained from three separate cultures.
[b] Incubation for 30 min. The media contained 2 μM bovine albumin and either 922,600 dpm of [1-^{14}C]EPA or 824,500 dpm of [1-^{14}C]arachidonic acid.
[c] Incubation of the cultures, after washing, for 5 min in 1 ml of medium containing 50 μM bovine albumin.
[d] Incubation for 5 min in 1 ml of medium containing 50 μM bovine albumin and 1 U of bovine thrombin.
[e] These values were calculated by subtracting the release obtained when the medium contained only albumin from that occurring when the medium contained both thrombin and albumin.

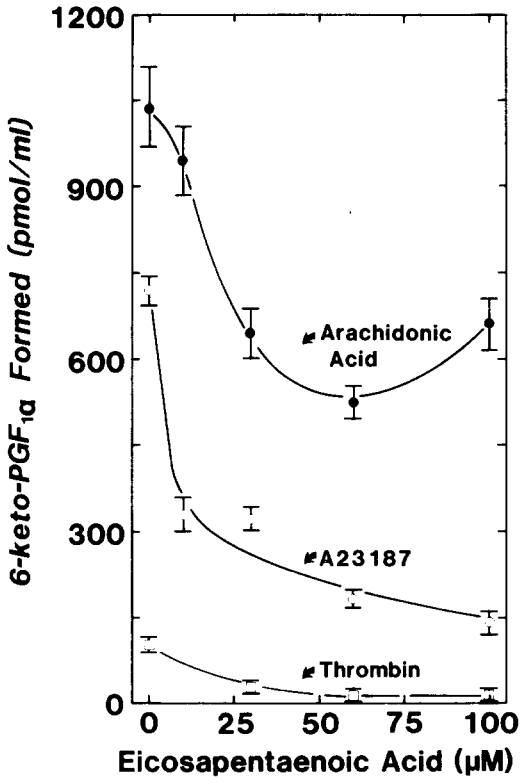

Fig. 2. The effect of an initial incubation with media containing supplemental EPA on the capacity of the endothelial cultures to produce PGI_2. Cultures were incubated for 20 hr in a medium containing 90 μM bovine serum albumin, 2.5% fetal bovine serum, and the amount of EPA shown on the figure. After incubation, the medium was removed and the cultures were washed with Dulbecco's phosphate-buffered saline, followed by a wash with this solution containing 50 μM albumin. They were then incubated for 5 min with either 1 U of thrombin, 10 μM calcium ionophore A23187, or 200 μM arachidonic acid combined with 50 μM albumin. PGI_2 production was measured by radioimmunoassay as 6-keto-$PGF_{1\alpha}$. Each value is the mean ± SE of results from three separate cultures.

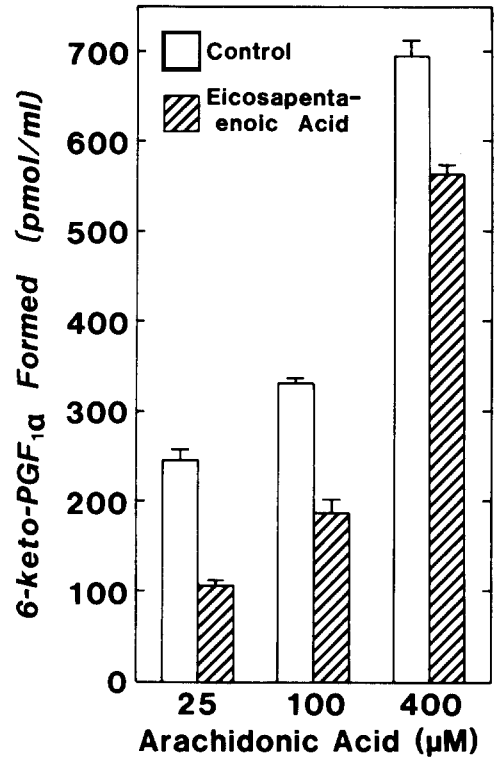

Fig. 3. Effect of arachidonic acid concentration on the degree of inhibition of PGI_2 production resulting from an initial incubation with EPA. Endothelial cell cultures were enriched for 20 hr with 100 μM EPA added to 90 μM bovine serum albumin and 2.5% fetal bovine serum. Corresponding control cultures were incubated for 20 hr in the same medium without supplemental EPA. After washing, the cultures were incubated for 10 min with arachidonic acid in a medium containing 50 μM albumin. The arachidonic acid concentration was increased from 25 to 400 μM. PGI_2 production was measured by radioimmunoassay as 6-keto-$PGF_{1\alpha}$. Each bar is the mean of values obtained from three separate cultures, and the overbar indicates the SE.

centration of arachidonic acid added. This is shown in **Fig. 3.** The cultures initially were exposed to 100 μM EPA for 24 hr. After washing, the cultures were incubated for 10 min with different amounts of arachidonic acid combined with 50 μM albumin. PGI_2 production increased as the concentration of arachidonic acid was raised. As compared with unsupplemented cultures, those previously enriched with EPA exhibited a 56% decrease in PGI_2 production when the test medium contained 25 μM arachidonic acid, 43% decrease when the medium contained 100 μM arachidonic acid, and only 19% decrease when it contained 400 μM arachidonic acid. This indicates that much of the inhibition produced by EPA can be overcome by increasing the availability of arachidonic acid.

Table 4 contains information regarding the specificity and time-dependence of the inhibitory effect of EPA on the capacity of the endothelial cultures to subsequently release PGI_2. The cultures were incubated with 100 μM of either EPA, linolenic acid, or docosahexaenoic acid. After washing, they were stimulated for 5 min with thrombin. There was no significant change in PGI_2 release between the unsupplemented control and the cultures

TABLE 4. Effect of n-3 polyunsaturated fatty acids on thrombin-stimulated PGI_2 production

Added Fatty Acid[a]	6-Keto $PGF_{1\alpha}$ Formed[b]	
	2 hr[c]	18 hr
	pmol/ml	
None	152 ± 11	133 ± 13
Eicosapentaenoic	124 ± 16	38 ± 2
Linolenic	149 ± 14	113 ± 11
Docosahexaenoic	153 ± 11	82 ± 5

[a] In each case, 100 μM fatty acid combined with 90 μM bovine serum albumin was added.

[b] Each value is the mean ± SE of results obtained from four separate cultures.

[c] Time of incubation of the cultures with the supplemental fatty acid.

enriched with any of these three fatty acids following 2 hr of supplementation. By contrast, a 70% reduction in thrombin-stimulated PGI_2 output occurred in those cultures supplemented for 18 hr with EPA. A 39% decrease occurred in the cultures supplemented for 18 hr with docosahexaenoic acid, but no significant reduction in PGI_2 output was observed in the cultures supplemented with linolenic acid.

Like EPA, prior incubation with a medium containing supplemental arachidonic acid inhibits the capacity of the endothelial cell cultures to produce PGI_2 in response to subsequent stimulation (30). This inhibition can be overcome, however, by incubating the cultures with arachidonic acid in the presence of a rapidly reversible cyclooxygenase inhibitor, ibuprofen (30). To determine whether the inhibitory effect of EPA also can be overcome if the incubation occurs in the presence of ibuprofen, cultures were enriched for 20 hr with increasing amounts of EPA together with 200 μM ibuprofen. After removal of the ibuprofen and washing, the cultures were stimulated for 5 min with thrombin. As seen in **Fig. 4,** an initial incubation with EPA in the presence of ibuprofen reduced subsequent thrombin-stimulated PGI_2 release, the maximum decrease being 85% relative to the unsupplemented control cultures. By contrast, an initial incubation with arachidonic acid in the presence of ibuprofen greatly increased the capacity of the cultures to subsequently release PGI_2 in response to thrombin.

An experiment with [1-^{14}C]arachidonic acid was done to determine whether the decrease in PGI_2 production that occurs when the cultures are enriched with EPA might be due to diversion of arachidonic acid into other prostaglandins. Unsupplemented control cultures incubated for 5 min with 550,000 dpm of [1-^{14}C]arachidonic acid converted 26,700 ± 2100 dpm (n = 3) into total prostaglandins. The distribution of the prostaglandin radioactivity was 63% as 6-keto-$PGF_{1\alpha}$, 30% as a product that co-migrates with prostaglandin $F_{2\alpha}$ and 7% as a product that co-migrates with prostaglandin E_2. When corresponding cultures were supplemented with 50 μM EPA for 20 hr prior to incubation for 5 min with 550,000 dpm [1-^{14}C]arachidonic acid, 12,700 ± 970 dpm (n = 3) was recovered in total prostaglandins, a 52% reduction, as compared with the unsupplemented control cultures. The distribution of radioactivity was 65% as 6-keto-$PGF_{1\alpha}$, 30% as a product that co-migrates with prostaglandin $F_{2\alpha}$, and 5% as a product that co-migrates with prostaglandin E_2. These results indicate that the EPA-induced decrease in PGI_2 production is not due to the diversion of arachidonic acid into other prostaglandins.

Formation of prostaglandins from EPA

Endothelial cultures were incubated with [1-^{14}C]EPA in order to determine whether this fatty acid is converted to prostaglandins. For comparison, corresponding cultures were incubated with [1-^{14}C]arachidonic acid. After 5 min, 485 ± 32 pmol of [1-^{14}C]arachidonate was converted to prostaglandins (n = 3), 364 ± 23 pmol of which was recovered as 6-keto-$PGF_{1\alpha}$. Under the same conditions, the cultures incubated with [1-^{14}C]EPA converted 39 ± 1 pmol to prostaglandins (n = 3), only 8% of the total produced by the cultures incubated with arachidonic acid. Of this, 30 ± 1 pmol was converted to a product that co-migrated with 6-keto-$PGF_{1\alpha}$.

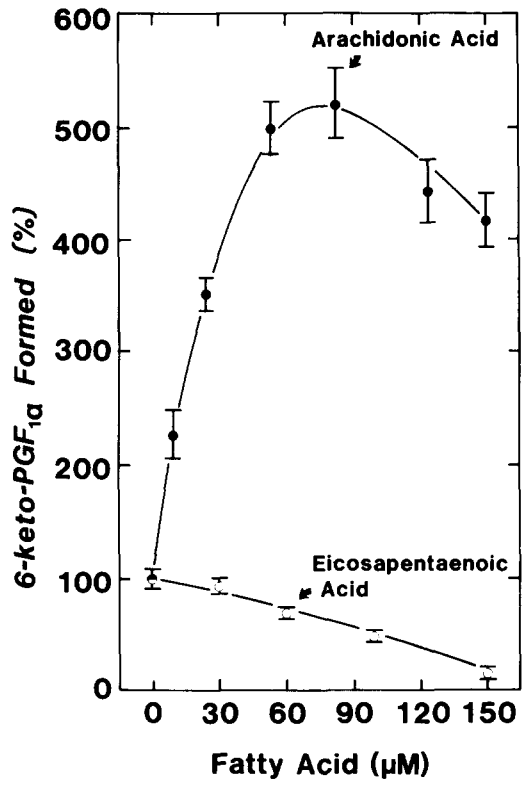

Fig. 4. Effects of an initial incubation with either supplemental EPA or arachidonic acid on PGI_2 production by endothelial cultures when the medium also contains ibuprofen. Cultures were incubated for 20 hr with either EPA or arachidonic acid in a medium containing 90 μM bovine serum albumin, 2.5% fetal bovine serum, and 200 μM ibuprofen. The concentration of supplemental fatty acid to which the cultures were exposed is shown on the abscissa. After these media were removed and the cultures were washed, they were incubated for 5 min with 1 U of thrombin. PGI_2 release was measured by radioimmunoassay as 6-keto-$PGF_{1\alpha}$. Each value is the mean ± SE of results from three separate experiments. The amounts of 6-keto-$PGF_{1\alpha}$ formed in the three cultures that did not contain supplemental fatty acid, shown as 100%, were 223, 210 and 145 pmol/ml.

DISCUSSION

Cultured human umbilical vein endothelial cells incorporate EPA present in the extracellular fluid and, under certain conditions, can convert appreciable amounts of linolenic acid to EPA. However, only a very small

amount of the linolenic acid uptake is converted to EPA under the usual conditions employed for endothelial cell culture, a medium containing 20% serum (18–21). EPA formation also decreased when the linolenic acid concentration was raised above the tracer range. These results are consistent with the findings of Rosenthal and Whitehurst (29). Although endothelial cells have the capacity to perform Δ6- and Δ5-desaturation of polyunsaturated fatty acids, these enzymes apparently are repressed or inhibited when either the serum or fatty acid concentration in the medium is raised. Since these reactions are reduced markedly when the medium contains as little as 20% serum, it seems unlikely that the conversion of linolenic acid to EPA occurs to any appreciable extent in the endothelium in vivo. Therefore, any EPA contained in the endothelial cells probably is taken up preformed from an extracellular source. The plasma EPA concentration ordinarily is very low, but it increases when the diet is high in n-3 polyunsaturates (2, 3). The present results suggest that, as has been reported for human platelets (2, 3), the endothelium may accumulate EPA under these conditions.

Rosenthal and Whitehurst (29) found no conversion of linolenic acid to docosahexaenoic acid and concluded that endothelial cells do not express a Δ4-desaturase activity. In our studies with [1-^{14}C]linolenic acid or [1-^{14}C]EPA, some radioactivity always eluted from the gas–liquid chromatography column in conjunction with the 22:6 peak. In neither case, however, was there any increase in the content of 22:6 in cellular phospholipids. It appears that either the radioactivity recovered as 22:6 is an artifact or, if actual conversion occurs, the amount produced is too small to increase measurably the 22:6 content of the phospholipids.

PGI_2 production decreased considerably when the endothelial cultures were enriched with EPA. This is consistent with the findings in rat aorta of Hornstra et al. (13) and Quadt and ten Hoor (31), and of Morita et al. in both aorta and smooth muscle cells (14). Conversely, others have reported that EPA either does not influence the conversion of arachidonic acid to PGI_2 by vascular tissue (10) or actually increases this conversion (12). One possibility is that these differences are related to variations in the peroxide content of the various tissue preparations. Culp, Titus, and Lands (32) have shown that the cellular peroxide level regulates cyclooxygenase activity and have concluded that, at physiological peroxide concentrations, EPA should inhibit prostaglandin synthesis from arachidonic acid. Our findings with endothelial cells, as well as those with smooth muscle cells (14), several vascular preparations (13, 31), and platelets (1–3, 6, 7), agree with this conclusion.

Lands and Byrnes (33) suggested that EPA inhibits prostaglandin production through two independent mechanisms, displacement of arachidonic acid from tissue phospholipids and competitive inhibition of cyclooxygenase activity. Both of these mechanisms appear to be operative in the endothelial cell. Enrichment with EPA was associated with a significant reduction in the arachidonic acid content of the cellular choline and ethanolamine glycerophospholipids. The phospholipid that supplies arachidonic acid for prostaglandin production has not yet been determined in endothelial cells. Although much of the recent data suggest that the inositol glycerophospholipids are the main source of intracellular arachidonic acid utilized for prostaglandin production (34–36), there is evidence in some systems for involvement of the choline and ethanolamine glycerophospholipids in this process (37–39). The lesser content of arachidonic acid in these phospholipids may account in part for the decreased PGI_2 production when the endothelial cells are stimulated with thrombin or calcium ionophore A23187. However, decreased PGI_2 output also occurs when arachidonic acid is supplied in the assay medium, suggesting that the conversion of arachidonic acid to PGI_2 is inhibited when the cells are enriched with EPA. Competition is suggested by the fact that the degree of inhibition is reduced as the concentration of arachidonic acid is raised. This observation is consistent with theoretical considerations; the effect of a competitive inhibitor should be overcome progressively by increasing substrate concentration. Based on the findings of Lands and Byrnes (33), it is likely that the inhibition occurs at the cyclooxygenase step. This is consistent with the finding that the conversion of radioactive arachidonic acid to several prostaglandins is reduced when the cells are enriched with EPA. Our data do not exclude the possibility, however, that EPA enrichment leads to an inhibition of other enzymes involved in prostaglandin synthesis; for example, enzymes that utilize prostaglandin H_2 such as PGI_2 synthase. If inhibition occurs at this step rather than at the cyclooxygenase reaction, several different synthases would have to be affected to account for the observation that the production of more than one prostaglandin is reduced. Phospholipase inhibition also would be consistent with a generalized decrease in prostaglandin production. Although the cells exhibited apparently normal phospholipase activity after EPA incorporation (Table 3), this study was done with tracer amounts of radioactive EPA and an incubation time of only 30 min. Whether amounts of EPA incorporation sufficient to appreciably alter cellular fatty acid composition would interfere with phospholipase activity was not tested.

Since both EPA and arachidonic acid are released when the endothelial cells are exposed to thrombin, inhibition probably also occurs when intracellular substrates are utilized for prostaglandin production. As in the endothelial cell, thrombin stimulates EPA release in platelets (1, 5), and a similar mechanism combining competitive inhibition and reduced phospholipid arachidonic acid content has

been suggested to explain the decrease in thromboxane A_2 production when platelets are enriched in EPA (1). The decrease in PGI_2 production was only about 20% after 2 hr of exposure to EPA, whereas it increased to 70% after 18 hr (Table 4). This probably is due to the fact that the EPA content of the cell phospholipids increased progressively during the first 24 hr of exposure to EPA and, concomitantly, the arachidonic acid content decreased. Presumably, the extent of the fatty acid compositional changes after 2 hr of exposure was insufficient to have a major effect on PGI_2 production.

Some [1-^{14}C]EPA was converted to a 6-keto-$PGF_{1\alpha}$-like material by the endothelial cultures, but the amount was less than 10% of that produced from arachidonic acid under comparable conditions. This low value is consistent with the conclusion of Lands and Byrnes (33), who predicted that arachidonic acid is utilized by cyclooxygenase at least five times better than EPA in the physiologic state. In agreement with our finding, EPA conversion to a PGI_3 has been observed in pieces of human umbilical vessels, but at a lesser rate than with arachidonic acid (10, 11). By contrast, EPA was found not to be converted to PGI_3 by murine arterial smooth muscle cells (14), rabbit aortic rings, heart or kidney (1). The apparent inconsistencies could be due to tissue or species differences. It must be stressed that, even though we find some conversion of EPA to a PGI-like material in the endothelial cultures, the amount produced relative to the production from arachidonic acid is so small as to be of questionable functional significance.

The main conclusion of this work is that PGI_2 production is reduced when endothelial cells are exposed to EPA. Morita et al. (14) have reached the same conclusion regarding the arterial smooth muscle cell. Therefore, increased EPA availability is likely to lower the capacity of arterial tissue to produce PGI_2, a substance that promotes vasodilation and prevents platelet aggregation (15). Although some PGI-like material is formed by the endothelial cells from EPA, the amount produced appears to be far too little to compensate for the decrease in PGI_2 formation. Because EPA also reduces thromboxane A_2 production by platelets, it has been suggested as possibly being useful as a preventive measure against the thrombotic complications of atherosclerotic cardiovascular disease (1, 2, 6–9). While this almost certainly would be beneficial, it appears that such treatment also might lead to a decrease in the production of PGI_2. Whether the benefits outweigh any deleterious effects that might result from a lesser capacity to produce PGI_2 remains to be determined.

We wish to thank Dr. Derek F. Stubbs, The Upjohn Company, Kalamazoo, Michigan, for supplying sodium ibuprofen. This work was supported by Arteriosclerosis Specialized Center of Research grant HL 14230 from the National Heart, Lung, and Blood Institute, National Institutes of Health.

Manuscript received 20 June 1983.

REFERENCES

1. Needleman, P., A. Wyche, L. Le Duc, S. K. Sankarappe, B. A. Jakschik, and H. Sprecher. 1981. Fatty acids as sources of potential "magic bullets" for the modification of platelet and vascular function. *Prog. Lipid Res.* **20:** 415–422.
2. Dyerberg, J., and H. O. Bang. 1979. Haemostatic function and platelet polyunsaturated fatty acids in Eskimos. *Lancet.* **ii:** 433–435.
3. Siess, W., B. Scherer, B. Böhlig, P. Roth, I. Kurzmann, and P. C. Weber. 1980. Platelet-membrane fatty acids, platelet aggregation, and thromboxane formation during a mackerel diet. *Lancet.* **i:** 441–444.
4. Needleman, P., A. Ray, 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.
5. McKean, M. L., J. B. Smith, and D. Ahern. 1981. Metabolism of 5,8,11,14,17-eicosapentaenoic acid by human platelets. *Prog. Lipid Res.* **20:** 435–438.
6. Whitaker, M. O., A. Wyche, F. Fitzpatrick, H. Sprecher, and P. Needleman. 1979. Triene prostaglandins: prostaglandin D_3 and icosapentaenoic acid as potential antithrombotic substances. *Proc. Natl. Acad. Sci. USA.* **76,** 5919–5923.
7. Needleman, P., M. O. Whitaker, A. Wyche, K. Watters, H. Sprecher, and A. Raz. 1980. Manipulation of platelet aggregation by prostaglandins and their fatty acid precursors: pharmacologic basis for a therapeutic approach. *Prostaglandins.* **19:** 165–181.
8. 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.
9. Jakubowski, J. A., and N. G. Ardlie. 1979. Evidence for the mechanism by which eicosapentaenoic acid inhibits human platelet aggregation and secretion—implications for the prevention of vascular disease. *Thromb. Res.* **16:** 205–217.
10. Dyerberg, J., and K. A. Jørgenson. 1980. The effect of arachidonic- and eicosapentaenoic acid on the synthesis of prostacyclin-like material in human umbilical vasculature. *Artery.* **8:** 12–17.
11. Dyerberg, J., J. A. Jørgensen, 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.
12. Hamazaki, T., A. Hirai, T. Terano, J. Sajiki, S. Kondo, T. Fujita, Y. Tamura, and A. Kumagai. 1982. Effects of orally administered ethyl ester of eicosapentaenoic acid (EPA; 20:5 ω-3) on PGI_2-like substance production by rat aorta. *Prostaglandins.* **23:** 557–567.
13. Hornstra, G., E. Christ-Hazelhof, E. Haddeman, F. ten Hoor, and D. H. Nugteren. 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.
14. 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.

15. Moncada, S. 1982. Prostacyclin and arterial wall biology. *Arteriosclerosis.* **2:** 193–207.
16. Marcus, A. J., B. B. Weksler, and E. A. Jaffe. 1978. Enzymatic conversion of prostaglandin endoperoxide H_2 and arachidonic acid to prostacyclin by cultured human endothelial cells. *J. Biol. Chem.* **253:** 7138–7141.
17. Weksler, B. B., C. W. Ley, and E. A. Jaffe. 1979. Stimulation of endothelial cell prostacyclin production by thrombin, trypsin and the ionophore A23187. *J. Clin. Invest.* **63:** 1089–1092.
18. Czervionke, R. L., J. C. Hoak, and G. L. Fry. 1978. Effect of aspirin on thrombin-induced adherence of platelets to cultured cells from the blood vessel wall. *J. Clin. Invest.* **62:** 847–856.
19. Jaffe, E. A., R. L. Nachman, C. G. Becker, and C. R. Minnick. 1973. Culture of human endothelial cells derived from umbilical cord veins. Identification of morphologic and immunologic criteria. *J. Clin. Invest.* **52:** 2745–2756.
20. 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.
21. Spector, A. A., J. C. Hoak, G. L. Fry, L. L. Stoll, C. T. Tanke, and T. L. Kaduce. 1981. Essential fatty acid availability and prostacyclin production by cultured human endothelial cells. *Prog. Lipid Res.* **20:** 471–477.
22. Spector, A. A., T. L. Kaduce, J. C. Hoak, and G. L. Fry. 1981. Utilization of arachidonic and linoleic acids by cultured human endothelial cells. *J. Clin. Invest.* **68:** 1003–1011.
23. Lees, M. B., and S. Paxman. 1972. Modification of the Lowry procedure for the analysis of proteolipid protein. *Anal. Biochem.* **47:** 184–192.
24. Folch, J. M., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
25. Morrison, W. R., and L. M. Smith. 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride–methanol. *J. Lipid Res.* **5:** 600–608.
26. Kaduce, T. L., A. A. Spector, and R. S. Bar. 1982. Linoleic acid metabolism and prostaglandin production by cultured bovine pulmonary artery endothelial cells. *Arteriosclerosis.* **2:** 380–389.
27. Czervionke, R. L., J. B. Smith, J. C. Hoak, G. L. Fry, and D. L. Haycraft. 1979. Use of a radioimmunoassay to study thrombin-induced release of PGI_2 from cultured endothelium. *Thromb. Res.* **14:** 781–786.
28. Salmon, J. A., D. R. Smith, R. J. Flowers, S. Moncada, and J. R. Vane. 1978. Further studies on the enzymatic conversion of prostaglandin endoperoxide into prostacyclin by porcine aorta microsomes. *Biochim. Biophys. Acta.* **523:** 250–262.
29. Rosenthal, M. D., and C. Whitehurst. 1983. Fatty acyl Δ6 desaturation activity of cultured human endothelial cells. Modulation by fetal bovine serum. *Biochim. Biophys. Acta.* **750:** 490–496.
30. 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.
31. Quadt, J. F. A., and F. ten Hoor. 1981. Effect of dietary α-linolenic acid on the production of a PGI-like substance of the isolated pulsating perfused rat aorta. *Prog. Lipid Res.* **20:** 581–585.
32. Culp, B. R., B. G. Titus, and W. E. M. Lands. 1979. Inhibition of prostaglandin biosynthesis by eicosapentaenoic acid. *Prostaglandin Med.* **3:** 269–278.
33. 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.
34. Rittenhouse-Simmons, S. 1979. Production of diglyceride from phosphatidylinositol in activated human platelets. *J. Clin. Invest.* **63:** 580–587.
35. Bell, R. L., and P. W. Majerus. 1980. Thrombin-induced hydrolysis of phosphatidylinositol in human platelets. *J. Biol. Chem.* **255:** 1790–1792.
36. Habenicht, A. J. R., J. A. Glomset, W. C. King, C. Nist, C. D. Mitchell, and R. Ross. 1981. Early changes in phosphatidylinositol and arachidonic acid metabolism in quiescent Swiss 3T3 cells stimulated to divide by platelet-derived growth factor. *J. Biol. Chem.* **256:** 12329–12335.
37. Bills, T. K., J. B. Smith, and M. J. Silver. 1977. Selective release of arachidonic acid from the phospholipids of human platelets in response to thrombin. *J. Clin. Invest.* **60:** 1–6.
38. Brockman, M. J., J. W. Ward, and A. J. Marcus. 1980. Phospholipid metabolism in stimulated human platelets. Changes in phosphatidylinositol, phosphatidic acid and lysophospholipids. *J. Clin. Invest.* **66:** 275–283.
39. Beaudry, G. A., L. King, L. W. Daniel, and M. Waite. 1982. Stimulation of deacylation in Madin-Darby canine kidney cells. Specificity of deacylation and prostaglandin production in 12-O-tetradecanoyl-13-acetate-treated cells. *J. Biol. Chem.* **257:** 10973–10977.