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Enzymes and Receptors of Prostaglandin Pathways with Arachidonic Acid-derived *Versus* Eicosapentaenoic Acid-derived Substrates and Products*◆

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Dietary fish oil containing ω3 highly unsaturated fatty acids has cardioprotective and anti-inflammatory effects. Prostaglandins (PGs) and thromboxanes are produced *in vivo* both from the ω6 fatty acid arachidonic acid (AA) and the ω3 fatty acid eicosapentaenoic acid (EPA). Certain beneficial effects of fish oil may result from altered PG metabolism resulting from increases in the EPA/AA ratios of precursor phospholipids. Here we report *in vitro* specificities of prostanoid enzymes and receptors toward EPA-derived, 3-series *versus* AA-derived, 2-series prostanoid substrates and products. The largest difference was seen with PG endoperoxide H synthase (PGHS)-1. Under optimal conditions purified PGHS-1 oxygenates EPA with only 10% of the efficiency of AA, and EPA significantly inhibits AA oxygenation by PGHS-1. Two- to 3-fold higher activities or potencies with 2-series *versus* 3-series compounds were observed with PGHS-2, PGD synthases, microsomal PGE synthase-1 and EP1, EP2, EP3, and FP receptors. Our most surprising observation was that AA oxygenation by PGHS-2 is only modestly inhibited by EPA (*i.e.* PGHS-2 exhibits a marked preference for AA when EPA and AA are tested together). Also unexpectedly, TxA_3 is about equipotent to TxA_2 at the TPα receptor. Our biochemical data predict that increasing phospholipid EPA/AA ratios in cells would dampen prostanoid signaling with the largest effects being on PGHS-1 pathways involving PGD, PGE, and PGF. Production of 2-series prostanoids from AA by PGHS-2 would be expected to decrease in proportion to the compensatory decrease in the AA content of phospholipids that would result from increased incorporation of ω3 fatty acids such as EPA.

North American and Western European diets have relatively high levels of ω6 fatty acids (*e.g.* linoleic acid (1, 2)). As a result, the most common highly unsaturated fatty acid is the C20 ω6 fatty acid, arachidonic acid (AA).[3] AA is present mainly at the *sn*2-position of membrane phospholipids. Humans ingesting fish oil enriched in ω3 fatty acids show increased amounts of eicosapentaenoic acid (EPA) in their membrane phospholipids and an approximately corresponding decrease in the level of AA. The ratio of ω3 EPA/ω6 AA in tissue phospholipids from human populations averages less than 0.1 (1, 2) but can be increased to almost 0.7 with palatable diets enriched in fish oil (3, 4). An increased dietary intake of fish oil is cardioprotective, anti-inflammatory, and anti-carcinogenic (2, 5–14).

The molecular basis for the health benefits of dietary fish oil is almost surely multifactorial. For example, ω3 fatty acids attenuate responses of T-cells (15) and macrophages (16) to agents working through cell surface receptors perhaps by changing the composition of membrane microdomains (17, 18). One ω3 fatty acid, docosahexaenoic acid (DHA), has been shown to be essential in the development and maintenance of neuronal functions including visual acuity. This may also be related to the ability of DHA to change the physical properties of membranes in a way that facilitates rhodopsin signaling (17, 19–22). Anti-arrhythmic effects of ω3 fatty acids may relate to their stabilizing effect on cardiac cell membranes and inhibition of the fast, voltage-dependent sodium and L-type calcium currents (12). Nonesterified polyunsaturated fatty acids, particularly EPA, can also influence transcription acting through peroxisomal proliferator-activated receptors and sterol response

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[3] The abbreviations used are: AA, arachidonic acid; PG, prostaglandin; PGDS, PGD synthase; mPGES-1, microsomal PGE synthase-1; PGHS, prostaglandin endoperoxide H synthase; COX, cyclooxygenase; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PRP, platelet-rich plasma; Tx, thromboxane; TxAS, thromboxane A synthase; HHTrE, 12(*S*)-hydroxy-5,8,10-heptadecatrienoic acid; HHTE, 12(*S*)-hydroxy-5,8,10-heptadecatetraenoic acid; hu, human; mu, murine; ov, ovine; IP, inositol phosphate; YPD, yeast extract/peptone/dextrose; YEL, yeast extract-sodium lactate; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; Ni-NTA, nickel-nitrilotriacetic acid; MES, 4-morpholineethanesulfonic acid.

element binding protein-1c, major transcription factors controlling lipid metabolism (2, 23, 24). Other proteins that can be activated directly by polyunsaturated fatty acids, and thus, whose activities might be altered by changes in EPA/AA ratios include protein kinase C (25), NADPH oxidase (26), and a two-pore domain K^+ channel (27). Polyunsaturated fatty acids such as AA can promote apoptosis but the mechanism is not known (28).

Finally, the eicosanoid pathways for lipid mediator formation; including the cyclooxygenase pathways, the 5-, 12-, and 15-lipoxygenase pathways, the P450 epoxygenase pathways, and non-enzymic oxidative pathways; are influenced by changes in EPA/AA ratios (29–35). Anti-thrombotic, anti-inflammatory, and anti-carcinogenic effects of ω3 fatty acids could result, at least in part, from their ability to attenuate the synthesis of specific eicosanoids and/or to alter the nature of the eicosanoid products formed or to serve as precursors of novel products such as isoprostanes and resolvins (32, 33, 35–38).

Prostanoids are synthesized via the cyclooxygenase pathway, most commonly from AA, in response to various hormones and physical stimuli (29). The pathway involves three stages: (*a*) mobilization of AA from membrane phospholipids by cytosolic phospholipase $A_2\alpha$ (cPLA$_2\alpha$) sometimes in conjunction with secretory sPLA$_2$s; (*b*) conversion of AA to the prostaglandin endoperoxide PGH_2 by prostaglandin endoperoxide H synthase-1 or -2 (PGHS-1 or -2) also known as cyclooxygenase-1 or -2 (COX-1 or -2); and (*c*) isomerization of PGH_2 to a "2-series" product, PGD_2, PGE_2-, $PGF_{2\alpha}$, PGI_2, or thromboxane A_2 (TxA_2), by specific synthases. Newly formed PGs exit cells and function primarily through G-protein-coupled receptors on neighboring or parent cells to elicit responses. Because PGs act at or near their sites of synthesis and are rapidly metabolized, they are considered to be "local" hormones. Importantly, EPA can serve as a substrate for PG formation generating "3-series" PG products including PGD_3, PGE_3, $PGF_{3\alpha}$, PGI_3, and TxA_3.

There is only limited biochemical information available on the specificities of the enzymes and receptors of the prostanoid pathways with EPA-derived *versus* AA-derived substrates and products. Here we report studies that address this topic.

EXPERIMENTAL PROCEDURES

Materials—U46619, $^{\Delta 17}$U46619, PGI_2, PGI_3, iloprost, SQ29548, AA, EPA, HHTrE, lipocalin PGD synthase (L-PGDS), and hematopoietic (H) PGDS were purchased from Cayman Chemicals (Ann Arbor, MI). [^{3}H]SQ29548 was purchased from PerkinElmer Life Sciences. [1-^{14}C]AA and [1-^{14}C]EPA were from American Radiolabeled Chemicals. [^{3}H]myoinositol and a cAMP assay kits were from Amersham Biosciences. SQ22536 was from Biomol. Cell culture materials were purchased from Invitrogen. Human fibrinogen, α-thrombin, and γIIa-thrombin were purchased from Hematologic Technologies, Inc. Collagen was obtained from Chronolog Corp. Complete protease inhibitor was from Roche Applied Science. BCA protein reagent was from Pierce. Restriction enzymes were from New England Biolabs, Inc. Ni-NTA was from Qiagen. All other materials were purchased from Fisher Scientific.

Expression, Purification, and Assay of PGHSs—Hexahistidine-tagged (His_6) ovine (ov) PGHS-1, murine (mu) murine PGHS-2, and human (hu) PGHS-2 were expressed in S21 insect cells and purified through Ni-NTA chromatography essentially as described previously (39–41). His_6-muPGHS-1 was expressed also in insect cells but was unstable following Ni-NTA chromatography so in the experiment using this enzyme, the supernatant from centrifugation of solubilized cell pellets was used for COX assays. Oxygen electrode assays for COX activity were performed as detailed in previous reports (39–41). COX assays of purified enzymes utilizing radio thin layer chromatography assays of PGH_2 or PGH_3 formation were performed using [1-^{14}C]AA and [1-^{14}C]EPA as described previously (41).

Preparation of Platelet-rich Plasma (PRP)—Platelets were obtained from normal human donors who had not taken medication during the 2 weeks prior to donation. Whole blood was drawn into 3.8% sodium citrate (1:9; citrate:blood). The blood was centrifuged at 180 × *g* for 10 min at room temperature, and PRP was transferred to a new tube. The remaining blood was centrifuged at 1000 × *g* for 10 min at room temperature to obtain PRP. For aggregation studies using PRP, the platelet count was determined on a Coulter counter (Model Z; Coulter Electronics, Hialeah, FL) and adjusted with HEPES-Tyrode's buffer (137 mM NaCl, 3 mM KCl, 12 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, 14.7 mM HEPES, 0.35% dextrose, and 0.35% bovine serum albumin, pH 7.4) to 2.2 to 2.5 × 10^8 platelets/ml. For preparation of washed platelets, human platelets in PRP were separated from plasma by gel filtration over Sepharose 2B columns in HEPES-Tyrode's buffer. The peak tubes were pooled, and the platelet count was adjusted to 2.5 × 10^8 platelets/ml before proceeding with platelet aggregation studies. Washed platelets (400 μl) were placed in a cuvette in the aggregometer and stirred at 37 °C. The integrity of the washed platelets was tested by their ability to be activated by collagen (1–5 mg/ml) and α-thrombin (3 nM).

PGDS Assays—PGDS activity was determined essentially as described previously (42, 43). First, PGH_2 or PGH_3 were prepared from 18 μM [1-^{14}C]AA or [1-^{14}C]EPA, respectively, by incubation for 20 s at room temperature with purified His_6-muPGHS-2 (30 unit) in 100 μl of 0.1 M Tris-Cl, pH 8.0, containing 2 mM phenol, 20 μM hematin, and γ-globulin (1 mg/ml). PGH_2/PGH_3 isomerization to PGD_2/PGD_3 was initiated by the addition of either lipocalin or hematopoietic PGDS (0.04 unit) premixed with 0.1 mM GSH, followed by incubation for 40 s at room temperature. Reactions were quenched by adding 500 μl of diethylether/methanol/0.2 M citric acid (30:4:1). After vortexing for 10 s, the reaction mixture was centrifuged at 1000 × *g* × 10 min at 4 °C. An aliquot of organic extract (100 μl) was separated by thin layer chromatography on a silica gel plate in ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (110:50:20:100). Regions of the plates migrating with PGD, PGH, and other products were scraped into vials and radioactivity quantified by liquid scintillation counting. One unit of PGDS enzyme represents 1 μmol of PGD_2/min at 25 °C in 100 mM Tris-HCl, pH 8.0, containing 1 mM GSH, 1 mg/ml γ-globulin, and 40 μM PGH_2.

Expression, Purification, and Assay of Human Microsomal PGE Synthase-1 (hu mPGES-1)—The human PGES cDNA (Invitrogen) was amplified using High Fidelity PCR kit (Invitrogen) with the 5′-primer (with BspHI) GAA TTC ATC ATG ATC CCT GCC CAC AGC CTG GTG A and the 3′-primer (with HindIII and His_6-tag) CAT CCA AGC TTG TCA GTG GTG GTG GTG GTG GTG GTG *CAG GTG GCG GGC CGC AAC*. The PCR product was purified with a QIAquick PCR purification kit (Qiagen). The pRMGsp expression vector[4] was digested by AflIII and XhoI and the amplified PGES PCR product was digested with BspHI and HindIII. The digested DNA was isolated by electrophoresis on a 1% agarose gel, and the DNA band was purified with a QIAquick gel purification kit (Qiagen). The digested and purified expression plasmid and His_6-hu mPGES-1 insert were ligated with T4 DNA ligase. The ligation sample was transformed into DH5-α competent cells, and plasmids from positive colonies were sequenced to confirm the expression construct pRMGsp-PGES-His_6.

The pRMGsp-PGES-His_6 DNA with the aid DNA pAL9 (digested by PstI) were transformed into freshly made *Schizosaccharomyces pombe* competent cells. After selection of positive colonies on MAA plates, the potential transformants were screened twice on yeast extract/peptone/dextrose medium (YPD)/G418 plates (containing G418 at 20 μg/ml). Positive single colonies were then grown in yeast extract-sodium lactate (YEL) medium (with 10 μg/ml G418) to make glycerol stocks, which were stored at −80 °C.

For expression of His_6-hu mPGES-1 in *S. pombe*, 5 ml of YEL medium (with 10 μg/ml G418) was inoculated with 200 μl of a glycerol stock culture and shaken for 24 h at 32 °C. The culture was transferred into 50 ml of fresh YEL/G418 medium and incubated at 32 °C for 48 h with shaking until the A_{600} was 10–12. The culture was then transferred into 1 liter of YPD medium (with 100 μg of G419/ml) and incubated at 32 °C with shaking until the A_{600} exceeds 20 (~48–60 h). The cells were harvested by centrifugation at 3000 × *g* for 15 min at 4 °C, and the cell pellet was stored at −80 °C.

After completely thawing the cell paste in ice water, 5 ml of lysis buffer (15 mM Tris-HCl, 250 mM sucrose, 0.1 mM EDTA, 1 mM reduced glutathione, pH 8.0) was added for per gram of cell pellet. The resuspended cell pellet was lysed using an Emulsi-Flex-C3 (at 20,000–25,000 p.s.i. with two passes). The cell lysate was centrifuged at 8000 × *g* for 20 min at 4 °C. The membrane-containing supernatant fraction was then centrifuged at ~200,000 × *g* for 1 h at 4 °C. The membrane pellet was resuspended in loading buffer (50 mM sodium phosphate, 300 mM NaCl, 10% glycerol, pH 8.0) and dodecyl maltoside (Anatrace) was added to a final concentration of 1%. The membrane fraction was stirred for 1 h at 4 °C and then centrifuged at 200,000 × *g* for 1 h at 4 °C. Imidazole was added to the supernatant fraction to a final concentration of 10 mM, and the mixture was loaded onto an Ni-NTA (Qiagen) column equilibrated with loading buffer supplemented with 10 mM imidazole; all column buffers contained 0.05% dodecyl maltoside. After loading, the column was washed with loading buffer containing 22 mM imidazole. The bound His_6-hu mPGES-1 was eluted using loading buffer containing 200 mM imidazole. Elution fractions containing His_6-hu mPGES-1 were pooled and concentrated using a 30 molecular weight cutoff Centricon spin concentrator.

Microsomal Preparations of TxA Synthase (TxAS)—Mouse (mu) TXAS was expressed in Sf21 insect cells as described previously (44, 45). The cells from a 250-ml culture were harvested after 4 days, collected, and washed twice with ice-cold phosphate-buffered saline (PBS) and stored at −80 °C. Cell pellets were thawed on ice and resuspended in 100 mM Tris-HCl pH 7.4, 1 mM EDTA and 1× Complete protease inhibitor. Cells were disrupted by sonication and centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant was then centrifuged at 100,000 × *g* for 1 h at 4 °C. The resulting pellet was homogenized in 10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, and 20% glycerol using a Dounce homogenizer and the protein concentration measured. The protein was used immediately for *in vitro* synthesis of TxA_2 or TxA_3.

PGE (EP), PGF (FP), and TxA/PGH (TP) Receptor Binding—HEK cell lines expressing various human PGE (EP2, EP3 (EP3II isoform (46)), EP4 (47, 48)), PGF (FP; (49)), PGI (IP; (50)), and TxA/PGH (TP; (51, 52)) receptors were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat inactivated fetal bovine serum, 250 μg/ml Geneticin, 200 μg/ml hygromycin, 100 μg/ml gentamicin and maintained at 37 °C with 5% CO_2. The cells were grown to 60% confluence and harvested from five 100-mm tissue culture dishes by scraping into the medium and centrifuged at 100 × *g* for 5 min. The cell pellets were washed once with ice-cold PBS, harvested, and stored at −80 °C until membranes were prepared for competitive binding assays.

The PCR was used to amplify the coding domain of the huEP1 receptor (nucleotides 1–1209; GenBank™ accession number L22647 (53)) from human kidney cDNA. The product encoding the huEP1 was purified by agarose gel electrophoresis and cloned into the EcoRV site of pcDNA3 to yield huEP1/pcDNA3. The sequence of huEP1 in huEP1/pcDNA3 was verified by DNA sequencing. huEP1/pcDNA3 encoding the huEP1 was transiently transfected into HEK293 cells using Lipofectamine 2000 according to the recommendation of the manufacturer, and cells were harvested 30 h post-transfection. Cell pellets were stored at −80 °C and membranes prepared from these cells were used to determine the relative affinities of PGE_2 *versus* PGE_3.

Membranes were prepared from HEK293 cells essentially as described by Ungrin *et al.* (54). Briefly, cell pellets were thawed on ice and resuspended in Buffer A (10 mM HEPES/KOH, pH 7.4, with 1 mM EDTA and 1× Complete protease inhibitor), disrupted by sonication, and centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant was centrifuged at 100,000 × *g* for 1 h at 4 °C. The pellet was homogenized in Buffer A, and aliquots of the suspended protein (50–100 μg) were used immediately for binding assays.

Binding assays were performed in 200 μl of 10 mM MES, pH 6.0, 1 mM EDTA and 10 mM $MgCl_2$. Binding isotherms were performed for [^{3}H]PGE_2, [^{3}H]$PGF_{2\alpha}$, or the TPA antagonist [^{3}H]SQ29548 to estimate K_d values for the different receptors with the cognate 2-series PG ligand. A concentration of ^{3}H-la-

[4] D. Sui and R. M. Garavito, unpublished results.

beled ligand corresponding to the K_d value for each receptor was then used in competition binding assays with PGE_2 *versus* PGE_3, $PGF_{2\alpha}$ *versus* $PGF_{3\alpha}$, or U46619 *versus* $^{\Delta 17}$U46619. Nonspecific binding was determined in the presence of 10 μM unlabeled ligand. Samples were incubated at 30 °C for 1 h and then filtered through Whatman GF/C glass filters. The filters were washed three times with cold MES buffer (without EDTA) and radioactivity measured by liquid scintillation counting. Receptor binding data were analyzed by nonlinear regression in Origin. Statistical analyses were performed using Student's *t* test and/or ANOVA.

cAMP Assays—HEK cell lines expressing EP2 and EP4 receptors that had been grown as described above in 6-well plates were treated for 15 min at 37 °C with fresh DMEM containing 50 mM isobutylmethylxanthine. Cells were then treated with various concentrations of PGE_2 or PGE_3 for an hour. The treatments were terminated by scrapping cells into 0.5 ml of TE (50 mM Tris-HCl, pH 7.5, containing 4 mM EDTA) and boiling for 8 min. After centrifuging the lysates, 50 ml of the supernatant (from about 10^5 cells) was used for cAMP analysis using an Amersham Biosciences cAMP assay system kit following the instructions of the manufacturer. The samples were quantified by scintillation counting and values for cAMP were calculated from the cAMP standard curve.

Assays of Inositol Phosphates (IPs)—Assay of IPs was performed by measuring receptor induced production of [^{3}H] IPs as described previously (55). Briefly, HEK293 cells expressing EP3 or FP receptors were grown in 24-well plates as described above and labeled by incubating overnight with 1 μCi of [^{3}H]myoinositol (Amersham Biosciences) per ml of DMEM. The cells were treated with 10 mM LiCl for 15 min prior to adding various concentrations of PGE_2/PGE_3 or $PGF_{2\alpha}$/$PGF_{3\alpha}$ for 1 h. Assays were terminated by adding 3 ml of chloroform/methanol/water (1:1:1) to each well. The whole cell lysates were collected and centrifuged, and the resulting aqueous phase was applied to a Dowex AG1-X8 anion exchange column (Bio-Rad) to remove unincorporated [^{3}H]myoinositol. The IPs were eluted with 0.2 M ammonium formate, 0.1 M formic acid and quantified by liquid scintillation counting.

Measurements of Intracellular Ca^{2+}—Intracellular Ca^{2+} concentrations were measured as described previously by Fisher *et al.* (56). Nontransfected HEK293 cells and huEP1 plasmid-transfected HEK293 cells were incubated with 1 μM Fura-2/AM (Invitrogen) for 15 min before adding various concentrations of PGE_2 or PGE_3. The Ca^{2+} signals were measured using a Shimadzu RF-5301 PC spectrofluorometer.

Platelet Aggregation—Aggregation assays were performed on a Chronolog dual-channel aggregometer at 37 °C. The assays were carried out with 360 μl of undiluted human PRP, and the compounds to be tested were added to obtain a final volume of 400 μl. The assay was measured for 3 min following platelet activation. Only PRP that was responsive to 20–40 nM γIIa-thrombin was used for experiments. Variable amounts of each aggregatory compound were added to PRP to determine the threshold concentration for platelet aggregation: U46619 or $^{\Delta 17}$U46619 (0.1 nM to 2 μM) and collagen (1–2 μg/ml). In some experiments, inhibition of aggregation induced by 2 μM U46619 was examined using different amounts of anti-aggregatory compounds: iloprost (0.1–10 nM) and PGI_2 or PGI_3 (10 pM to 2 μM); flurbiprofen was added at 100 μM. All compounds were diluted from a stock solution in organic solvent into PBS, pH 7.5, prior to the collection of blood, except for PGI_2 and PGI_3, which were prepared by removing a solid chemical stock vial from −80 °C (stored for less than 1 week) and dissolving in PBS less than 15 min before adding to PRP. The stability of each prostacyclin was checked by comparing the anti-aggregatory activity of 1 nM at the end of the experiment to that of the beginning of the experiment.

Platelet Aggregation by TxA_2 Versus TxA_3—An aliquot of PRP (370 μl) was placed into a cuvette in the aggregometer while stirring at 37 °C. The TxA_2 and TxA_3 were prepared just before adding to PRP. In an Eppendorf tube, AA or EPA was added to a final concentration of 5 μM in reaction buffer (100 mM Tris-HCl, pH 7.4, 1 mM phenol, and 10 μM heme) containing 260 units of COX-2 to initiate the synthesis of PGH_2 or PGH_3. The sample was vortexed for 20 s. Then, 30 μl of TxA microsomal protein (~500 μg) was added to produce TxA_2 or TxA_3 in a final volume of 100 μl. The mixture was vortexed for 10 s, at which time 30 μl was removed and added to the 370 ml of PRP. Immediately, prior to the experiments using PRP, parallel reactions were performed with ^{14}C-labeled AA or EPA and the products quantified by radio thin layer chromatography as described above. This provided an accurate estimate of the various products being added in each case to the PRP.

RESULTS

Specificities of PGHS-1 and PGHS-2 with AA and EPA—As shown in Fig. 1, purified ovPGHS-1 and muPGHS-2 oxygenate AA with comparable catalytic efficiencies at concentrations of ≥1 μM AA where reasonably precise O_2 electrode measurements of enzyme activity can be performed. ovPGHS-1 is essentially inactive with EPA while muPGHS-2 can use EPA with about 30% of the efficiency of AA in the range of 1–100 mM.[5] A solubilized preparation of muPGHS-1 expressed in baculovirus showed qualitatively similar results to those shown for ovPGHS-1 (data not shown); muPGHS-1 was unstable in our hands, and so we were unable to analyze purified enzyme. Although ovPGHS-1 was not active with low concentrations of EPA, significant activity (~10% of that with AA) was observed when 15 μM 15-hydroperoxyeicosatetraenoic acid was added to the reaction mixtures (data not shown). Purified huPGHS-2 also showed results very similar to those in Fig. 1 for muPGHS-2 (data not shown) when tested under essentially identical enzyme and substrate conditions.

Results similar to those illustrated in Fig. 1 have been reported by Kulmacz and co-workers using ovPGHS-1 (57, 58). Moreover, the results with purified and semipurified enzymes are consistent with studies comparing the utilization of AA *versus* EPA by microsomal huPGHS-1 and huPGHS-2 (59), where, under optimal conditions, huPGHS-1 is 5% as active with EPA as with AA while huPGHS-2 is 25–30% as active with EPA as

[5] Critical micelle concentrations for AA and EPA determined in 0.1 M sodium phosphate, pH 7.6. using fluorescence assays (*i.e.* with 1 mM *N*-phenyl-1-naphthylamine) were approximately 62 and 210 mM, respectively.

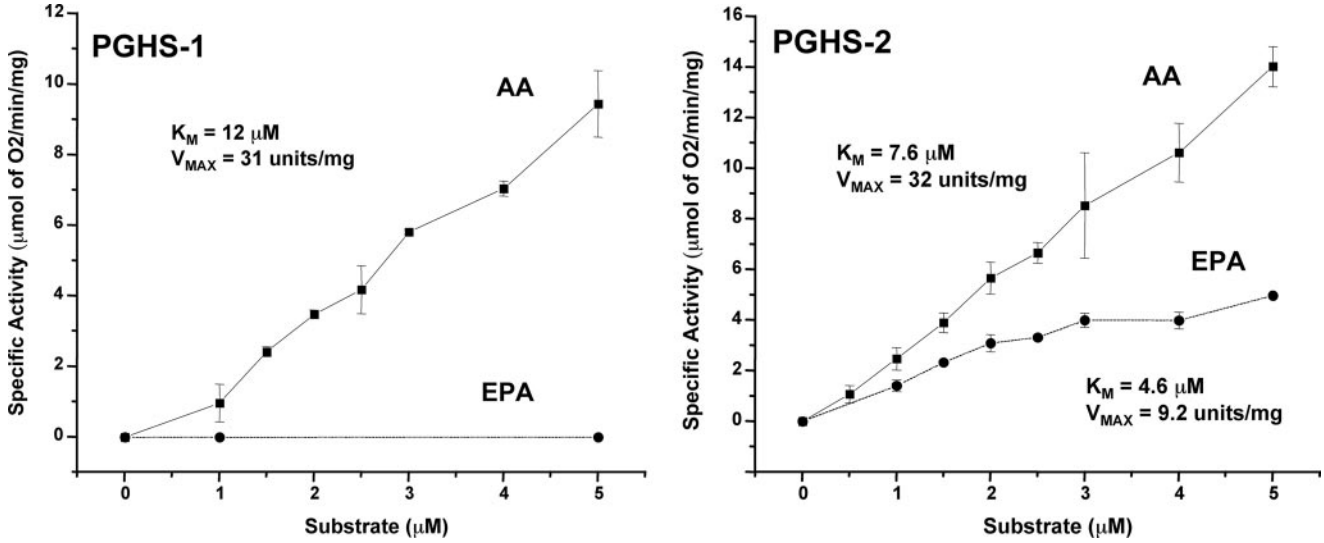


FIGURE 1. **COX activities of ovPGHS-1 (*left panel*) and muPGHS-2 (*right panel*) with AA and EPA.** Specific activities were measured using purified His_6-tagged native ovPGHS-1 (~8 μg) or His_6-tagged muPGHS-2 (ca. 8 μg). Assays were performed on an O_2 electrode using standard COX assays with the indicated concentrations of substrates.[5] Shown in this figure are results obtained with fatty acid substrate concentrations of 1–5 μM; however, K_m and V_{max} values were determined using fatty acid substrate concentrations of 1–100 μM. *Circles*, AA; *squares*, EPA. Each data point represents a total of four assays involving two separate enzyme preparations, and *error bars* represent mean ± S.D.

AA. It also is clear that EPA can be oxygenated by PGHS-1 in intact cells in a manner that is peroxide dependent (60).

EPA and AA have similar K_m values with PGHS-1 and PGHS-2 (Fig. 1 and (57, 59)), and so EPA would be expected to compete with AA for oxygenation. EPA/AA competition has been shown previously with PGHS-1 (61), and the results in Fig. 2 confirm these findings. Half-maximal inhibition occurs with equimolar AA *versus* EPA. Essentially identical results were also obtained with a solubilized preparation of His_6-muPGHS-1. AA is about a 10 times better substrate than EPA for ovPGHS-1 *in vitro*, and as shown in Fig. 3, inhibition of oxygenation reflects primarily inhibition of AA oxygenation. A 5-fold excess of EPA caused 40% inhibition of [1-^{14}C]AA oxygenation by ovPGHS-1. This result is similar but not identical to that of Fig. 2, which shows about 75% inhibition at these concentrations of AA plus EPA. As expected, [1-^{14}C]EPA was a poor substrate for PGHS-1; however, EPA oxygenation was augmented slightly by the presence of AA. Again, this is probably because hydroperoxide is being generated when AA is present along with EPA in the reaction mixtures and hydroperoxides potentiate EPA oxygenation (59, 60, 62).

In contrast to the results obtained with PGHS-1, EPA was a relatively poor inhibitor of AA oxygenation by PGHS-2 (Fig. 2). For example, at equimolar AA and EPA concentrations, the first point at which there was a statistically significant decrease in the rate of oxygenation with muPGHS-2, there was only a 10% decrease in O_2 consumption and even with a 5-fold excess of EPA there was less than a 20% decrease in oxygenase activity. Based on the kinetic constants for muPGHS-2 (Fig. 1) and huPGHS-2 for AA and EPA tested individually, one would expect about a 35% lower oxygenation rate with 20 μM AA plus 20 μM EPA and a 60% decrease in the rate with 20 μM AA plus 100 μM EPA. To examine this inconsistency, we incubated purified enzymes with [1-^{14}C]AA or [1-^{14}C]EPA with and without unlabeled competing substrate and measured the formation of radioactive PGH_2 or PGH_3 (plus HHTE (12(*S*)-hydroxy-5,8,10-heptadecatetraenoic acid) a degradation product of PGH_3) using radio thin layer chromatography (Fig. 3). When 20 μM unlabeled AA was added to reaction mixtures containing 20 μM [1-^{14}C]EPA, oxygenation of EPA by PGHS-2 was inhibited by 70–90% (Fig. 3). In contrast, with 20 μM [1-^{14}C]AA and 100 μM unlabeled EPA there was only a modest inhibition (~10%) of AA oxygenation (Fig. 3, *lower panel*).

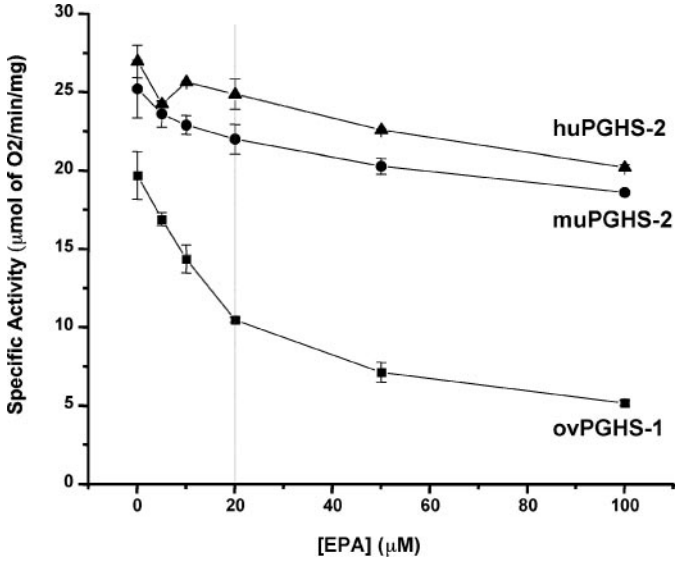


FIGURE 2. **Inhibition of COX activities of ovPGHS-1, muPGHS-2, and huPGHS-2 with AA and EPA.** Enzyme activity was measured using a standard COX oxygen electrode assay with 6–8 μg of His_6-tagged purified proteins, 20 μM AA, and the indicated concentrations of EPA as described under "Experimental Procedures" (39). Duplicate samples were assayed, and experiments with three different enzyme preparations yielded similar results. *Error bars* represent mean ± S.D.

Thus, EPA acts as an effective inhibitor of AA oxygenation by PGHS-1 but not PGHS-2, and indeed PGHS-2 shows a marked and unanticipated preference for AA when presented with a mixture of AA and EPA. The basis for the uneven competition between AA and EPA with PGHS-2 is not clear. It may involve

half of sites activity with PGHS-2 (40). In the case of PGHS-2 but not PGHS-1, binding of certain fatty acids to one COX site may facilitate oxygenation of AA bound to the other site.

In brief, our results with PGHSs show that (*a*) AA is an equally good substrate for PGHS-1 and PGHS-2; (*b*) EPA is a poorer substrate than AA for both PGHS-1 and PGHS-2 and a particularly poor substrate for PGHS-1; (*c*) EPA is an efficient inhibitor of AA oxygenation by PGHS-1 but not PGHS-2; and (*d*) in the presence of EPA, PGHS-2 shows a marked preference for AA.

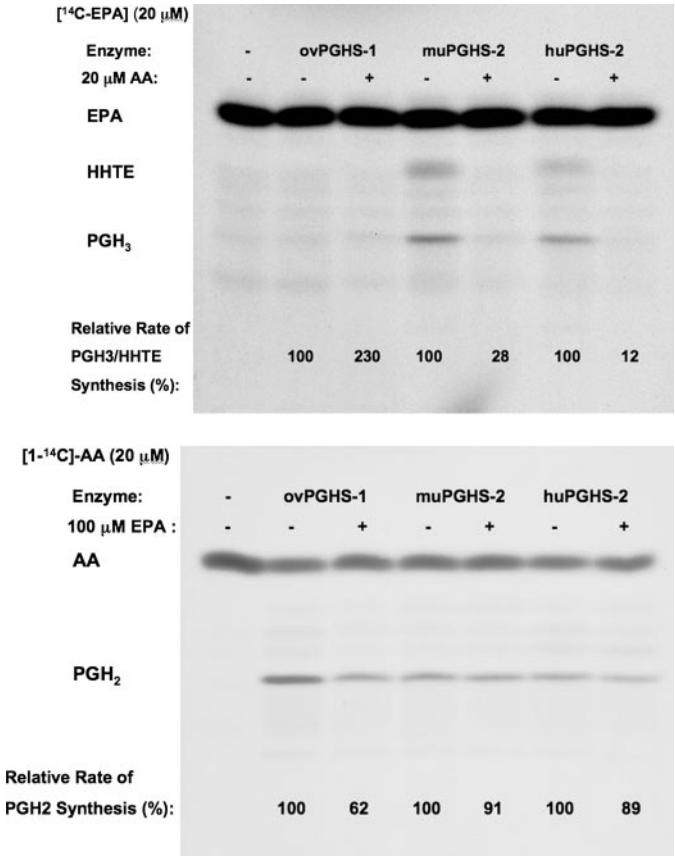


FIGURE 3. **Oxygenation of [1-^{14}C]EPA and [1-^{14}C]AA in the presence and absence of unlabeled AA or unlabeled EPA.** Radio thin layer chromatography assays were performed as described under "Experimental Procedures" (41). The indicated substrates were mixed with 0.5 μg (~12 units) of the purified His$_6$-tagged PGHSs and the reactions continued for 30 s. Products were extracted, separated, and visualized by autoradiography. The thin layer plates were subsequently scraped and the amounts of radioactivity associated with the substrates and products determined by scintillation counting and used to compute the relative rates indicated in the figure.

Specificities of Lipocalin PGDS, Hematopoietic PGD Synthase (hPGDS), and Microsomal PGES-1 (mPGES-1) toward PGH_2 Versus PGH_3—Table 1 shows data obtained in estimating the specificities of hPGDS, lPGDS, and mPGES-1 with PGH_2 *versus* PGH_3. PGH_2 and particularly PGH_3 are unstable, and so they were generated *in situ* quantitatively from AA or EPA using an excess of purified PGHS-2 and then a PGDS or PGES was added immediately and the reactions continued for 20–40 s. PGH_3 was found to be less stable than PGH_2, so it was necessary to add more EPA than AA in generating the endoperoxides so that the PGH_2 and PGH_3 concentrations were about the same when a PGDS or PGES was added. The reactions were terminated before 20% of the PGH was consumed, and the reactions were performed with amounts of enzyme that provided approximately linear product formation with time. Somewhat different V_{max} and K_m values have been reported for each of the various PGD and PGE synthases we tested (63–68). Because of this and the technical difficulties associated with multiple assays with unstable substrates and limited amounts of enzymes, we elected to use an endoperoxide substrate concentration in the range of 5 μM for all of our assays, because as noted earlier,[5] 5 μM PGH_2 or PGH_3 would likely be as high a concentration as would be encountered by a PGDS or PGES in an intact cell. With all these provisos, the human versions of H-PGDS, L-PGDS, and mPGES-1 were all more than 3-fold less active with PGH_3 than with PGH_2.

Quantitative data comparing the specificities of various prostanoid biosynthetic enzymes with AA *versus* EPA derived substrates is summarized in Table 2.

PGE and PGF Receptor Specificities—Membranes from HEK cell lines expressing various PGE (EP) and PGF (FP) receptors were used to determine the relative affinities of 2- *versus* 3-series PGE and PGF (Table 3). Except for the EP4 receptor, the affinity of each receptor was significantly greater for the 2-series than the 3-series PGs. The most dramatic difference was with the FP receptor, which had a 78-fold higher affinity for $PGF_{2\alpha}$ than $PGF_{3\alpha}$.

Fig. 4 shows the potencies of 2- *versus* 3-series PGs in eliciting second messenger formation via the EP and FP receptors. Differences were significant with EP1, EP2, EP3, and FP receptors as determined by ANOVA for EP1 ($p < 0.001$), EP2 ($p <$

TABLE 1

Specificities of human hematopoietic and lipocalin PGD synthases and microsomal PGE synthase-1 toward PGH_2 *versus* PGH_3

Human hematopoietic PGD synthase (H-PGDS; 0.04 unit (1 μg/assay)) and lipocalin PGDS (L-PGDS; 0.04 unit (18 μg/assay)) PGD synthases were from Cayman Chemical Co. Purified, solubilized His$_6$ mPGES-1 was expressed and purified as indicated under "Experimental Procedures" and 0.67–1 μg used for the assays presented in the table. [1-^{14}C]PGH_2 or [1-^{14}C]PGH_3 was prepared by incubation of 18 μM [1-^{14}C]AA or [1-^{14}C]EPA for 20 or 40 s. Hematopoietic or lipocalin PGDS or mPGES-1 was then added and the incubations performed for 40 s for PGDSs or 20 sec for mPGES-1 under conditions in which the rate of conversion to product (PGD or PGE) was approximately linear with time and added enzyme. [1-^{14}C]PGH_2 and [1-^{14}C]PGH_3 were generated *in situ* with purified muPGHS-2 or huPGHS-2 and H-PGDS, L-PGDS, or PGES was added to initiate the reactions in a final volume of 0.1 ml. Products were extracted, separated at 4 °C by thin-layer chromatography, and quantified by scintillation counting. Values in parentheses are numbers normalized for the indicated starting PGH_2 concentration.

PG synthase	PGH_2	PGH_3	PGD_2 or PGE_2	PGD_3 or PGE_3	PGD_2/PGD_3 or PGE_2/PGE_3
	nmol	*nmol*	*nmol*		
H-PGDS (1 μg)	8.0	4.4 (8.0)	0.51	0.063 (0.11)	4.6
H-PGDS (1 μg)	3.8	3.8	0.26	0.043	6.0
H-PGDS (1 μg)	3.8	4.7 (3.8)	0.21	0.035 (0.028)	7.5
L-PGDS (18 μg)	3.8	4.7 (3.8)	0.056	0.021 (0.017)	3.3
mPGES-1 (0.67 μg)	6.0	3.2 (6.0)	0.13	0.021 (0.039)	3.2
mPGES-1 (1.0 μg)	4.4	4.5	0.069	0.025	2.7

TABLE 2
Specificities of PG biosynthetic enzymes with AA- *versus* EPA-derived substrates

Enzyme	AA-derived substrates		EPA-derived substrates		Ref.
	K_m	Rel. rates	K_m	Rel. rates	
$cPLA_2$	$k_{cat}/K_m = 0.5$		Est. $k_{cat}/K_m = 0.5$		74–76
$sPLA_2$	Kinetic values are highly context dependent; mechanism of reaction does not permit discrimination among acyl groups.				79–81
ovPGHS-1	12 μM	31 units/mg	No activity without added hydroperoxide; <10% of activity with hydroperoxide; K_m similar to that of AA		57, 59, 83, 87; Fig. 1
muPGHS-2	7.6 μM	32 units/mg	4.6 μM	9.2 units/mg	57, 59; Fig. 1
H-PGDS	0.5 mM	$k_{cat} = 21\ s^{-1}$	17% activity with 5 μM PGH_3 *vs.* PGH_2		63, 64; Table 1
L-PGDS	14 μM	$k_{cat} \sim 50\ s^{-1}$	30% activity with 5 μM PGH_3 *vs.* PGH_2		65, 66; Table 1
mPGES-1	17 μM	$K_{cat} \sim 50\ s^{-1}$	30% activity with 5 μM PGH_3 *vs.* PGH_2		67, 68; Table 1
mPGES-2	28 μM	3.3 μmol/min/mg	ND		120
cPGES	14 μM	190 μmol/min/mg	ND		109
PGFS	Several enzymes proteins catalyzing the formation of PGF from PGH_2 have been reported; it is not clear which are physiologically important.				103, 104
PGI synthase	30 μM	$k_{cat} \sim 5\ s^{-1}$	About the same activity with PGH_3 and PGH_2		45, 71
TxA synthase	22 μM	$k_{cat} = 27\ s^{-1}$	About the same activity with PGH_3 and PGH_2		71

TABLE 3
EP and FP receptor specificities for PGE_2 *vs.* PGE_3 and $PGF_{2\alpha}$ *vs.* $PGF_{3\alpha}$
Membranes were prepared from HEK293 cell lines that stably express the human EP2, EP3, EP4, and FP receptors essentially as described by Ungrin *et al.* (54) as detailed under "Experimental Procedures."

Ligand	$IC_{50} \times 10^{-9}$ M for ligand binding to receptor				
	EP1	EP2	EP3	EP4	FP
PGE_2 or $PGF_{2\alpha}$	15 ± 6.2[a]	5.3 ± 0.86[a]	7.7 ± 1.6[a]	4.9 ± 1.4	2.3 ± 0.70[a]
PGE_3 or $PGF_{3\alpha}$	110 ± 31	20 ± 5.3	37 ± 8.7	17 ± 11	180 ± 110
Relative affinities (PG_3 *vs.* PG_2)	7.3	3.8	4.8	3.5	78

[a] Denotes significant difference between 2- and 3-series as determined by Student's *t* test. All binding assays were performed with duplicate samples with at least three different membrane preparations.

0.005), EP3 ($p < 0.0014$), and FP ($p < 0.0001$); ANOVA indicated no difference with the EP4 receptor ($p < 0.054$). In all cases the differences in potencies were less than the differences in binding affinities. However, it is important to note that, with the possible exception of the EP4 receptor, 3-series PGs were partial agonists. Quantitative data on receptor potencies are summarized in Table 4.

TPα Receptor Specificity toward U46619 and $^{\Delta 17}$U46619—Using membranes from HEK293 cells expressing the huTPα receptor, we determined the equilibrium dissociation constant for the TPα antagonist [^{3}H]SQ29548 to be 9 μM. Equilibrium competition binding assays with the PGH_2/TxA_2 and PGH_3/TxA_3 analogues U46619 and $^{\Delta 17}$U46619, respectively, were used to measure displacement of 5 μM [^{3}H]SQ29548 (Fig. 5). The IC_{50} values for U46619 and $^{\Delta 17}$U46619 were identical (*i.e.* 200 and 210 nM, respectively).

Comparison of U46619 and $^{\Delta 17}$U46619 Activation of Platelet Aggregation—PRP from human donors was treated with U46619 or $^{\Delta 17}$U46619 in amounts ranging from 0.1 to 2 μM to measure the potency of each compound in platelet aggregation (Fig. 6). The threshold concentrations ranged from 0.5 to 0.8 μM for U46619 and from 0.7 to 1 μM for $^{\Delta 17}$U46619 for platelets from four donors. For individual donors, the relative potencies of the compounds were similar, with the threshold concentration of $^{\Delta 17}$U46619 consistently around 1.2-fold greater than that of U46619. To confirm the specificities of the analogues for the TP receptor, the platelets were incubated first with TP antagonist SQ29548 (1 μM) before addition of the diene or triene analogue (2 μM) or of collagen (2 μg/ml). SQ29548 completely blocked aggregation by either U46619 or $^{\Delta 17}$U46619 and inhibited collagen-induced aggregation by ~50% (data not shown).

Effects of TxA_2 Versus TxA_3 on Platelet Aggregation—Previous studies had suggested that TxA_3 was essentially inactive in platelet aggregation (69, 70), while our results with $^{\Delta 17}$U46619 suggested that TxA_3 would be pro-aggregatory. This assessment raised the possibility that the $^{\Delta 17}$U46619 analogue behaves differently than authentic TxA_3. Because TxAs have very short half lives, we developed a system for synthesizing TxA_2 or TxA_3, which could then be added immediately to platelets. In brief, AA or EPA were treated with excess huCOX-2 to convert the fatty acids quantitatively to their respective endoperoxides. Excess microsomal TxAS was then added to quantitatively convert PGH_2 or PGH_3 to TxA_2 or TxA_3, respectively; TxAS is reported not to discriminate between PGH_2 and PGH_3 (71). An aliquot of the reaction mixture was immediately added to PRP and platelet aggregation was monitored. The amounts of the various products formed from AA and EPA by huPGHS-2 and TxAS were monitored in parallel reactions using [1-^{14}C]AA or [1-^{14}C]EPA.

A representative experiment is shown in Fig. 7. Reaction 2, with a concentration of 78 nM TxA_2, induced irreversible aggregation. However, Reaction 4, which contained 45 nM TxA_3, caused only a small reversible aggregation. When added to

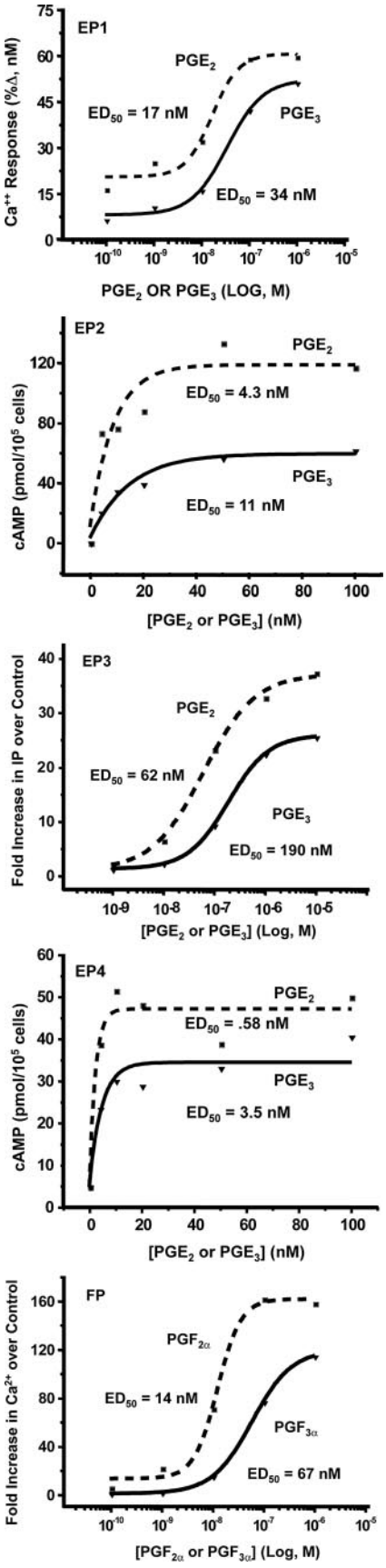


FIGURE 4. **Potencies of 2-*versus* 3-series PGs in eliciting second messenger formation by various EP receptors and the FP receptor.** HEK cells expressing the indicated EP1, EP2, EP3, EP4, and FP receptors were used to measure changes in cAMP or IP formation, or Ca^{2+} mobilization with the indicated concentrations of PGE_2, PGE_3, $PGF_{2\alpha}$ or $PGF_{3\alpha}$. Details of the experimental protocols are presented under "Experimental Procedures." All assays were performed in duplicate or triplicate with at least three cell preparations and data analyzed using ANOVA.

human PRP, PGH_3 was found to isomerize to PGD_3 with a $t_{½}$ of 90 s, unlike PGH_2 which has a $t_{½}$ of 350 s. PGD_3 is also reported to be somewhat more potent at inhibiting aggregation than PGD_2 (70), both of which cause an increase in cyclic AMP. To block the putative inhibitory effects of any PGD_3, 1 μM adenylate cyclase inhibitor SQ22536 was added to the PRP prior to the addition of Reaction 5. This unmasked an aggregatory effect equal to that of the TxA_2 reaction (Reaction 2).

Both PGH_2 and PGH_3 are ligands of the TP receptor. However, neither the diene nor the triene endoperoxide produced in the PGHS-2-only reactions 1 and 3 (Fig. 7) was generated at sufficient concentrations to induce irreversible aggregation. Thus, residual endoperoxide in the reactions containing TxAS could not be responsible for the irreversible aggregation seen in Reactions 2 or 5.

Thromboxane and HHTrE are reported to be produced in equimolar amounts by TxAS (71). However, in our *in vitro* reaction, HHTrE and HHTE were produced at approximately twice the concentration of their respective thromboxanes. The effect of HHTrE on platelet aggregation was investigated by adding 1 μM HHTrE to PRP alone or prior to the addition of either 2 μM U46619 or 2 μg/ml collagen. HHTrE neither induced nor inhibited platelet aggregation (results not shown). Likewise, up to 100 μM malondialdehyde, another side product of the TxAS reaction, had no effect on platelet aggregation (results not shown). The results of studies with the TPa receptor and platelet aggregation suggest that TxA_2 or TxA_3 are approximately equipotent.

Comparison of PGI_2 and PGI_3 as Inhibitors of Platelet Aggregation—PGI_2 or PGI_3 (0.1–2 μM) was added to PRP, immediately followed by 2 μM U46619. Preliminary experiments with several donors were performed to optimize experimental conditions, including the stabilization of the prostacyclins and determination of the approximate threshold concentrations of each compound, before proceeding to perform dose response measurements with three donors (Fig. 8). The initial slope of each curve in Fig. 8 was measured and expressed as the percent inhibition of aggregation *versus* PGI concentration. The average IC_{50} values were 0.92 $\pm$ 0.28 nM and 1.30 $\pm$ 0.18 nM for PGI_2 and PGI_3, respectively. Thus, the potencies of PGI_2 and PGI_3 in inhibiting platelet aggregation are approximately the same confirming earlier results (69).

DISCUSSION

The goal of the studies reported here was to compare the specificities and potencies of PG biosynthetic enzymes and receptors toward AA-derived, 2-series *versus* EPA-derived, 3-series substrates and products. We reason that this new information will contribute to understanding whether any of the reported beneficial health effects of dietary ω3 fish oil fatty acids are mediated through PG pathways. Our biochemical results along with those of others are summarized in Fig. 9 and Tables 2 and 4.

TABLE 4
PG receptors and their affinities or potencies with AA- *vs*. EPA-derived PGs

Receptor	EC_{50} 2-series PG	EC_{50} 3-series PG	Second messenger	Cell/Tissue	Ref.
	*n*M	*n*M			
DP1	109	64	G_s, cAMP	Platelets	70, 111
DP2	7	8	G_i, Ca^{2+}	Eosinophil	111
EP1	17	34[a]	G_q, Ca^{2+}	EP1 HEK cell	Table 3, Fig. 4; PGE_3 is partial agonist
EP2	4.3	11[a]	G_s, cAMP	EP2 HEK cell	Table 3, Fig. 4; PGE_3 is partial agonist
EP3	62	190[a]	G_i, reduced cAMP, IP increases	EP3 HEK cell	Table 3, Fig. 4; PGE_3 is partial agonist
EP4	0.58	3.5	G_s, cAMP	EP4 HEK cell	Table 3, Fig. 4; PGE_3 is partial agonist
FP	14	67[a]	G_q, Ca^{2+}	FP HEK cell	Table 3, Fig. 4; $PGF_{3\alpha}$ is partial agonist
IP	0.92	1.3	G_s, cAMP	Platelets	Fig. 8
TP	650 for U44619	850 for Δ17-U46619	G_q, Ca^{2+}	Platelets	Fig. 6

[a] Denotes significant difference. ANOVA ($p < 0.05$).

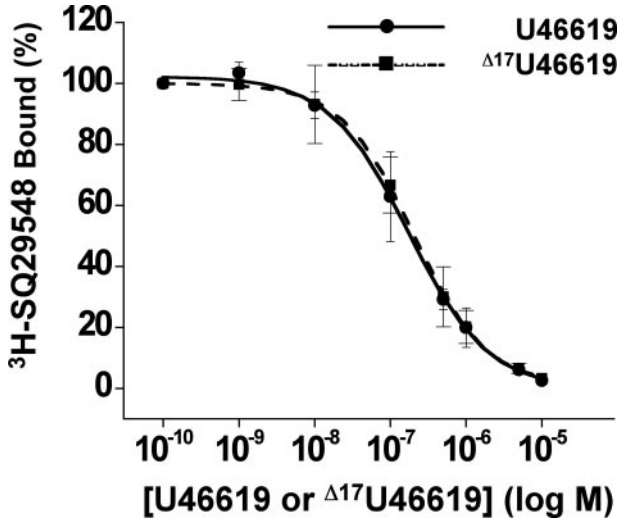


FIGURE 5. **Binding of U46619 and $^{\Delta 17}$U46619 to the huTPα receptor.** An equilibrium competition binding assay was performed for U46619 and $^{\Delta 17}$U46619 *versus* [^{3}H]SQ29548 (5 μM) as the radioligand. The average K_d and B_{max} values for [^{3}H]SQ29548 binding to TPa/HEK293 microsomes from three experiments was 10.2 ± 3.1 nM and 3800 ± 980 fmol/mg of protein, respectively. The average IC_{50} values for U46619 and $^{\Delta 17}$U46619 were 200 and 210 nM, respectively, from an average of three experiments depicted in the figure.

Phospholipases— $cPLA_2\alpha$ is the key phospholipase involved in AA release in most PG forming cells (72). Previous studies have shown that $cPLA_2\alpha$ exhibits specificity toward AA and EPA esterified at the 2-position of phospholipids in comparison to other 2-position acids such as linoleate and oleate (73–76). Moreover, although EPA- and AA-containing phospholipids are equally good substrates for $cPLA_2\alpha$, DHA-containing phospholipids are essentially inactive with $cPLA_2\alpha$ (76–78). Certain $sPLA_2$ forms can also participate in PG biosynthesis (79, 80). Because of the nature of the interaction with its substrates, $sPLA_2$ does not discriminate among 2-position acyl groups (81). In short, neither $cPLA_2$ nor $sPLA_2$ appears to differentiate between the acyl chains of AA *versus* EPA.

PGHS-1 and PGHS-2—PGHS-1 and PGHS-2 both exhibit specificity toward AA *versus* EPA. As reported previously by others and us, both enzymes have very similar K_m and V_{max} values with AA (57, 59). PGHS-2 oxygenates EPA at about 30% of the rate of AA. Purified PGHS-1 is not active with EPA unless an exogenous hydroperoxide is added to the reaction mixture. This is a consequence of the higher hydroperoxide requirement of PGHS-1 *versus* PGHS-2 (57, 82, 83). PGHS-1 present in platelets cells does oxygenate exogenously supplied EPA albeit at a low rate in the presence of alkyl hydroperoxides (60). It is not clear whether the hydroperoxide concentration in cells is usually sufficient to support EPA oxygenation or whether there are differences in hydroperoxide concentrations among cell types (57, 84).

The behavior of PGHS-1 with AA plus EPA is consistent with the kinetic properties of the enzyme determined with AA and EPA individually; thus, EPA is a reasonably good inhibitor of AA oxygenation by PGHS-1 as was originally reported by Lands and co-workers (61). A comparison of the crystal structures of EPA and AA with PGHS-1 suggests that EPA prefers to bind in a catalytically incompetent conformation in the PGHS-1 cyclooxygenase site and competes with AA for binding (85–87).

PGHS-1 mediated biological events include platelet aggregation and parturition (88, 89) and certain types of acute inflammation (79, 90, 91). Cellular events involving PGHS-1 may be dampened when EPA/AA ratios in phospholipids are increased. At an EPA/AA ratio of 1.0, one would expect that there would be 50% less AA to be mobilized from phospholipids by $cPLA_2$ and that PGHS-1 would function at only 50% of maximal efficiency because of inhibition of AA oxygenation by EPA; however, this may well be an oversimplification because the concentrations of enzymes, receptors, and substrates in intact cells are unknown.

One of our most surprising observations was that PGHS-2 preferentially oxygenates AA when EPA and AA are tested together. The results observed when PGHS-2 is mixed with EPA plus AA cannot be explained based on the simple kinetic properties of PGHS-2 with AA or EPA individually. The biochemical basis for the selectivity of PGHS-2 for EPA *versus* AA when the substrates are together may relate to the half of sites activity of the enzyme (40). One possibility is that EPA binds one of the two cyclooxygenase sites of the PGHS-2 dimer and elicits an allosteric effect on the other cyclooxygenase site causing it to preferentially bind and oxygenate AA. If this is true and also applicable to any fatty acid, it could explain why PGHS-2 can preferentially oxygenate AA at low substrate concentrations when AA represents a small part of the available fatty acid pool in cells (92–94). A situation like this could occur in so-called late phase PG synthesis when an $sPLA_2$ is the operative phospholipase (95).

PGs are importantly involved in inflammation (79, 91, 96–98), and in this context PGHS-2 is the most important PGHS isoform (99, 100). Based on our biochemical studies, a decrease in the formation of 2-series PGs via PGHS-2 would be

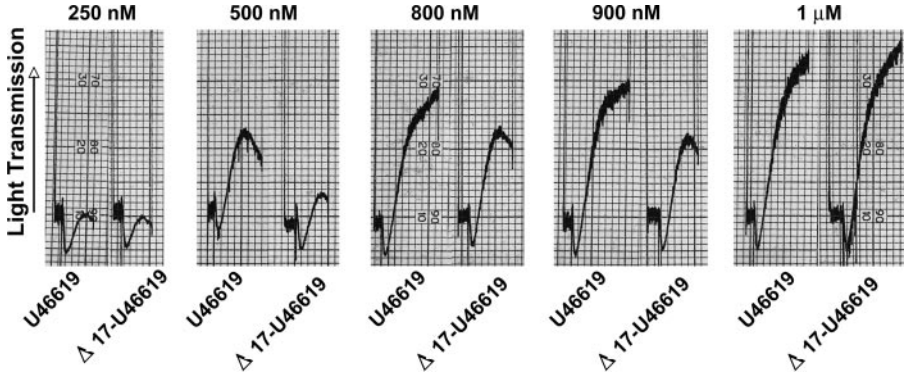
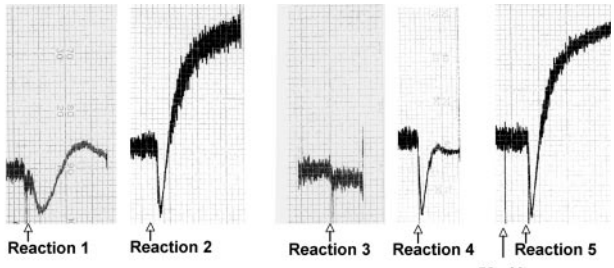


FIGURE 6. **Comparison of potencies of U46619 and $^{\Delta 17}$U46619 for platelet aggregation.** Human PRP (2.25×10^8 platelets per 0.4 ml) was treated with various concentrations of either U46619 or $^{\Delta 17}$U46619 as indicated, and platelet aggregation as indicated by the change in light transmission was recorded on an aggregometer. Shown is a representative result of four different donors. The concentration at which irreversible aggregation occurred for $^{\Delta 17}$U46619 was 1.2 times higher than for U46619 with platelets from each donor.



Concentrations of reactants and products (nM):

Reaction:	1	2		3	4	5
TxS:	-	+	TxS:	-	+	+
AA	2.3	3	EPA	13	23	23
HETEs	25	34	$^{\Delta 17}$HETEs	95	145	145
HHTrE	20	199	HHTE	15	105	105
PGH_2	209	11	PGH_3	135	31	31
TxA_2	0	78	TxA_3	0	45	45

FIGURE 7. **Platelet aggregatory properties of AA- *versus* EPA-derived COX-2 and TXAS products.** Enzyme reactions were initiated by adding 5 μM AA (Reactions 1 and 2) or EPA (Reactions 3, 4, and 5) to a reaction mixture containing 750 units of purified His_6-tagged huPGHS-2. A microsomal huTXAS preparation (540 mg of protein) (Reactions 2, 4, and 5) or microsomal buffer (Reactions 1 and 3) was added and the sample vortexed for an additional 10 s. An aliquot (30 μl) of each reaction mixture was immediately added to PRP and platelet aggregation was measured. The adenylate cyclase inhibitor SQ22536 was added to the PRP 1 min prior to addition of the reaction mixture for Reaction 5. To calculate the concentrations of products from the COX-2/TXAS reactions that were added to PRP, [1-^{14}C]AA, or [1-^{14}C]EPA was used in place of the unlabeled substrate. The radiolabeled products were separated by TLC and the bands corresponding to fatty acid, HETE, (12-hydroxyheptadecatrienoic acid), HHT, PGH, and TxB were scraped and quantified by liquid scintillation counting and the final concentration of each product (nM) in the PRP was calculated. The concentration of TxA_2 or TxA_3 was corrected for degradation to TxB_2 or TxB_3 during the 10 s incubation as described under "Experimental Procedures."

expected to occur only to the extent that AA levels in phospholipids were decreased by ω3 fatty acids supplanting AA.

PGD, PGE, PGF, PGI, and TxA Synthases—There are seven different synthases reportedly involved in the conversion of PG endoperoxides to what are considered to be the biologically active PGs. Including the results from the present studies, there are now data on the specificities toward PGH_2 *versus* PGH_3 for H-PGDS, L-PGDS, mPGES-1, PGIS, and TXAS (Fig. 9 and Table 2). Still lacking is information on cPGES (101) and mPGES-2 (101, 102) and various putative NADPH-dependent and GSH-dependent PGFSs (103, 104).

PGDSs and mPGES-1 are about one third as active with PGH_3 as PGH_2. mPGES-1 does play a role in inflammation (67, 96, 97, 105, 106), and in principle, elevated levels of EPA could increase PGH_3 production and decrease PGH_2 formation, and the net effect would be decreased formation of PGE_2 with less than a corresponding increase in PGE_3. However, PGHS-2 appears to be the most relevant enzyme in inflammation and, as discussed above, increases in EPA have a relatively modest effect on the formation of PGH_2. PGE formation can occur via PGHS-1 and mPGES-1 (107). There are functions such as salt and water metabolism in the kidney that involve these two enzymes, and renal PGE_2 synthesis is diminished with no detectable production of PGE_3 in rats fed diets having elevated levels of fish oil (108).

PGIS and TXAS are reported to be similarly reactive with PGH_2 and PGH_3 (45, 71). This suggests that any effects of changes in tissue EPA/AA levels on PGI and TxA formation would occur primarily at the level of PGHSs and not PGIS or TXAS.

Prostanoid Receptors—There are nine G-protein-linked PG receptors. Previous comparisons of receptor specificities for the 2- *versus* 3-series PGs had been performed for the DP1 (70), DP2 (110, 111), EP1 (54), TP (69), and IP (69, 112) receptors. In all cases except for the TP receptor, there was little or no difference in the potencies of the 2- *versus* 3-series PGs. We performed both binding measurements and measurements of receptor potencies for all of the human receptors except the IP and DP receptors. In the case of the IP receptor, we analyzed potencies of purified PGI_2 *versus* PGI_3 using human platelets.

The EP1, EP2, and EP3 receptors bound less well and were less responsive to PGE_3 than PGE_2. As recently reviewed by Narumiya and coworkers (113), each of these receptor subtypes participate in a large number of functions each of which has the potential to be affected by increased tissue EPA/AA levels. However again, it should be noted that functions most likely to be affected are those that would be mediated via PGHS-1 and mPGES-1.

The FP receptor is known to be involved in parturition. Mice lacking $cPLA_2$ (114, 115), PGHS-1 (88, 89), or the FP receptor (116) have failures of parturition. Interestingly, this is also a characteristic of essential fatty acid deficiency that can be overcome with omega-6 but not omega-3 fatty acids (117–119). This could be accounted for by the low activity of PGHS-1 with EPA and the low potency of $PGF_{3\alpha}$ with the FP receptor. It is not clear what enzyme is responsible for $PGF_{2\alpha}$ formation *in vivo*, so we did not examine the PGF synthases that have been described (103) for their specificities toward PGH_2 *versus* PGH_3.

An unexpected observation in our studies of PG receptors was that TxA_3 is almost as active as TxA_2 with the TP receptor. Earlier studies indicating that TxA_3 is inactive in platelet aggre-

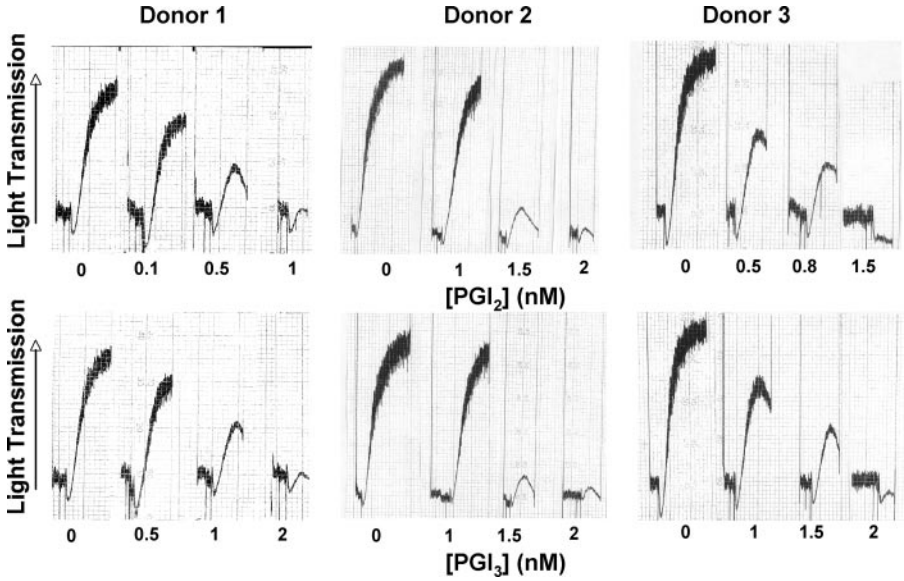


FIGURE 8. **Comparison of anti-aggregatory potencies of PGI_2 and PGI_3.** Human PRP from three donors was treated with the indicated concentrations of either PGI_2 or PGI_3 for 10 s, followed by treatment with 2 μM U46619 to induce aggregation. Aggregation was measured as an increase in light transmission using a platelet aggregometer. *Upper panels* are PGI_2, and *lower panels* are PGI_3. Agonist was introduced at points indicated by the *arrows*.

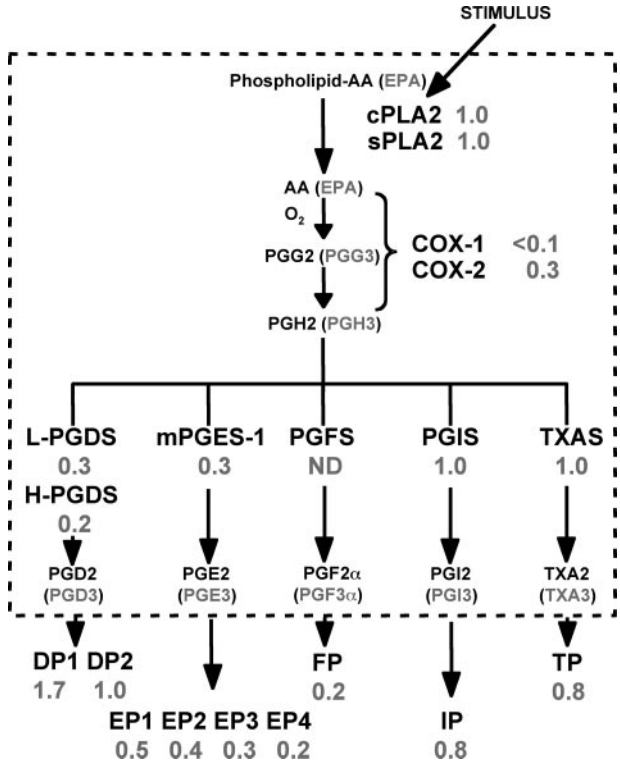


FIGURE 9. **Effectiveness of AA-derived *versus* EPA-derived substrates and products with enzymes and receptors of the PG pathway.** Abbreviations: TXAS, thromboxane A synthase; DP, EP, FP, IP, and TP are receptors for PGD, PGE, PGF, PGI, and TxA/PGH, respectively. *ND*, not determined.

gation (69) were probably compromised by the formation of PGD_3 from PGH_3 and PGD_3 being a potent anti-aggregatory compound (70). In general, PGH_3 appears to be significantly less stable than PGH_2 in the aqueous systems used for our enzyme assays; PGH_3 is rapidly converted to HHTE and malondialdehyde whereas spontaneous conversion of PGH_2 to the homologous products is relatively slow. This is apparent in Fig. 3 where there is an accumulation of HHTE but not HHTrE.

To the extent that we have discussed our biochemical data in the context of the biological changes seen with dietary fish oil, we have assumed simple linear relationships based on K_m, V_{max}, and EC_{50} values for the various enzymes and receptors. All of these values were obtained under optimal *in vitro* conditions. Obviously, what occurs *in vivo* cannot yet be predicted with any certainty because the ratios of enzymes and receptors to substrates and agonists involved in PG signaling may well be different *in vivo* (89). There may also be other eicosanoid mediators, including those derived from omega-3 fatty acids that are importantly involved in PG signaling (34, 35).

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