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 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 AA when EPA and AA are tested together. Also unexpectedly, TxA3 is equipotent to TxA2 at the TP 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). AA is present mainly at the sn2-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
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 epoxynogenase 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 A₂ (cPLA₂) sometimes in conjunction with secretory sPLA₂s; (b) conversion of AA to the prostaglandin endoperoxide PGH₂ 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 PGH₂ to a "2-series" product, PGD₂, PGE₂, PGF₂α, PGI₂, or thromboxane A₂ (TXA₂), by specific syntheses. 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 PGD₃, PGE₃, PGF₃α, PGI₃, and TXA₃.

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, Δ₁₂ U46619, PGI₂, PGI₃, iloprost, SQ29548, AA, EPA, HHT, lipocalin PGD synthase (L-PGDS), and hematopoietic (H) PGDS were purchased from Cayman Chemicals (Ann Arbor, MI). [³H]SQ29548 was purchased from PerkinElmer Life Sciences. [¹⁴C]AA and [¹⁴C]EPA were from American Radiolabeled Chemicals. [³H]myoinositol and a cAMP assay kits were from Amersham Biosciences. SQ22536 was from Biomat. Cell culture materials were purchased from Invitrogen. Human fibrinogen, α-thrombin, and γIa-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 (His₆) 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). His₆-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 PGH₃ or PGH₄ formation were performed using [¹-¹⁴C]AA and [¹-¹⁴C]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 NaHCO₃, 0.34 mm Na₂HPO₄, 14.7 mm HEPES, 0.35% dextrose, and 0.35% bovine serum albumin, pH 7.4) to 2.2 to 2.5 × 10⁸ 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 × 10⁸ 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, PGH₂ or PGH₃ were prepared from 18 µM [¹-¹⁴C]AA or [¹-¹⁴C]EPA, respectively, by incubation for 20 s at room temperature with purified His₆-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). PGHS₂/PGD₃ isomerization to PGD₂/PGD₄ 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 diethyl ether/methanol/0.2 M citric acid (30:4:1). After washing 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 PGD₂/min at 25 °C in 100 µM Tris-HCl, pH 8.0, containing 1 mM GSH, 1 mg/ml γ-globulin, and 40 µM PGH₂.
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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 BspHII) GAA TTC ATC ATG ATC CCT GCC CAC AGC GTG GTG A and the 3′-primer (with HindIII and His6-tag) CAT CAA AGC TGG TCA GTG GTG GTG GTG GTG CAG GTG GCC GCC GCC CAC A. The PCR product was purified with a QIAquick PCR purification kit (Qiagen). The pRMGsp expression vector 4 was digested by AfIII and XhoI and the amplified PGES PCR product was digested with BspHII 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 purified expression plasmid and His6-hu mPGES-1 insert were ligated with T4 DNA ligase. The ligation solution was transformed into DH5α competent cells, and plasmids from positive colonies were sequenced to confirm the expression construct pRMGsp-PGES-His6.

The pRMGsp-PGES-His6 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 His6-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 A600 was 10−12. The culture was then transferred into 1 liter ofYPD medium (with 100 μg of G419/ml) and incubated at 32 °C with shaking until the A600 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 EmulsiFlex-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 ~100,000 × 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 His6-hu mPGES-1 was eluted using loading buffer containing 200 mM imidazole. Elution fractions containing His6-hu mPGES-1 were pooled and concentrated using a 30 molecular weight cutoff Centricon spin concentrator.

Microsomal Preparations of TxA Synthase (TxA5)—Mouse (mu) TxA5 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% glycercol using a Dounce homogenizer and the protein concentration measured. The protein was used immediately for in vitro synthesis of TxA2 or TxA3.

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 (FP3); (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% CO2. 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; GenBankTM 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 PGE2 versus PGE2.

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 MgCl2. Binding isotherms were performed for [3H]PGE2 [3H]PGF2α, or the TPA antagonist [3H]SQ29548 to estimate Kd values for the different receptors with the cognate 2-series PG ligand. A concentration of [H-la-
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 $^{31}$H-U46619. Non-specific binding was determined in the presence of 10 $\mu$M unlabelled ligand. Samples were incubated at 37 °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 was 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 scraping 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$^7$ 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 [H]$^3$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 Ci of [H]$^3$H]myoinositol (Amersham Biosciences) per ml of DMEM. From the cAMP standard curve.

**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 $\geq$1 $\mu$M 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 $\mu$M. 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 $\mu$M 15-hydroperoxyeicosatetraenoic acid was added to the reaction mixtures (data not shown). Purified huPGHS-2 showed results very similar to those shown for ovPGHS-1 (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
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 [1-14C]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, [1-14C]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 $\mu$M AA plus 20 $\mu$M EPA and a 60% decrease in the rate with 20 $\mu$M AA plus 100 $\mu$M EPA. To examine this inconsistency, we incubated purified enzymes with [1-14C]AA or [1-14C]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 $\mu$M unlabeled AA was added to reaction mixtures containing 20 $\mu$M [1-14C]EPA, oxygenation of EPA by PGHS-2 was inhibited by 70–90% (Fig. 3). In contrast, with 20 $\mu$M [1-14C]AA and 100 $\mu$M unlabeled EPA there was only a modest inhibition (~10%) of AA oxygenation (Fig. 3, lower panel).

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.

Specificities of Lipocalin PGDS, Hematopoietic PGD Synthase (hPGDS), and Microsomal PGES-1 (mPGES-1) toward PGH₂ Versus PGH₃—Table 1 shows data obtained in estimating the specificities of hPGDS, lPGDS, and mPGES-1 with PGH₂ versus PGH₃. PGH₂ and particularly PGH₃ 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. PGH₃ was found to be less stable than PGH₂, so it was necessary to add more EPA than AA in generating the endoperoxides so that the PGH₂ and PGH₃ 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 PGH₂ or PGH₃ would likely be as high a concentration as would be encountered by a PGDS or PGE synthase 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 PGH₃ than with PGH₂.

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

PGEnzymes and Receptors

PG Enzymes and Receptors

**FIGURE 3. Oxygenation of [1-14C]EPA and [1-14C]AA in the presence and absence of unlabeled AA or unlabeled EPA.** 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 His₆-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.

**TABLE 1**

**Specificities of human hematopoietic and lipocalin PGD synthases and microsomal PGE synthase-1 toward PGH₂ versus PGH₃**

| PG synthase | PGH₂ | PGH₃ | PGD₂ or PGE₂ | PGD₃ or PGE₃ | PGD₂/PGD₃ or PGE₂/PGE₃ |
|-------------|------|------|--------------|--------------|------------------------|
| H-PGDS (1 μg) | 8.0  | 4.4  | 8.0          | 4.4 (8.0)    | 0.51                   |
| H-PGDS (1 μg) | 3.8  | 3.8  | 3.8          | 3.8          | 0.26                   |
| H-PGDS (1 μg) | 3.8  | 4.7  | 3.8          | 4.7 (3.8)    | 0.21                   |
| L-PGDS (18 μg) | 3.8  | 4.7  | 3.8          | 4.7 (3.8)    | 0.21                   |
| mPGES-1 (0.67 μg) | 6.0  | 3.2  | 6.0          | 3.2 (6.0)    | 0.13                   |
| mPGES-1 (1.0 μg) | 4.4  | 4.5  | 4.4          | 4.5          | 0.069                  |

Values in parentheses are numbers normalized for the indicated starting PGH₂ concentration.
PG Enzymes and Receptors

TABLE 2

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

| Enzyme       | AA-derived substrates | EPA-derived substrates |
|--------------|-----------------------|------------------------|
|              | $K_m$                 | Rel. rates             | $K_m$                 | Rel. rates |
|              | $k_{cat}/K_m$ = 0.5   |                        | $k_{cat}/K_m$ = 0.5   |            |
| cPLA$_2$     | 12 $\mu$m             | 31 units/mg            | 4.6 $\mu$m            | 9.2 units/mg |
| sPLA$_2$     |                       |                        |                       |            |
| ovPGHS-1     | 7.6 $\mu$m            | 32 units/mg            | 4.6 $\mu$m            | 9.2 units/mg |
| muPGHS-2     | 0.5 mM                | $k_{cat} = 21 s^{-1}$  | 4.6 $\mu$m            | 9.2 units/mg |
| H-PGDS       | 3.0 $\mu$m            | $k_{cat} = 50 s^{-1}$  | 4.6 $\mu$m            | 9.2 units/mg |
| L-PGDS       | 17 $\mu$m             | $k_{cat} = 50 s^{-1}$  | 4.6 $\mu$m            | 9.2 units/mg |
| mPGES-1      | 17 $\mu$m             | $k_{cat} = 50 s^{-1}$  | 4.6 $\mu$m            | 9.2 units/mg |
| mPGES-2      | 28 $\mu$m             | 3.3 $\mu$mol/min/mg    | 4.6 $\mu$m            | 9.2 units/mg |
| cPGES        | 14 $\mu$m             | 190 $\mu$mol/min/mg    | 4.6 $\mu$m            | 9.2 units/mg |
| PGFS         |                       |                        | 4.6 $\mu$m            | 9.2 units/mg |
| PGI synthase | 30 $\mu$m             | $k_{cat} = 5 s^{-1}$   | 4.6 $\mu$m            | 9.2 units/mg |
| TXA synthase | 22 $\mu$m             | $k_{cat} = 27 s^{-1}$  | 4.6 $\mu$m            | 9.2 units/mg |

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            |
| PGE$_2$ or PGF$_{2\alpha}$ | 15 $\pm$ 6.2*    | 5.3 $\pm$ 0.86*  | 7.7 $\pm$ 1.6*    | 4.9 $\pm$ 1.4    | 2.3 $\pm$ 0.70* |
| PGE$_3$ or PGF$_{3\alpha}$    | 110 $\pm$ 31      | 20 $\pm$ 5.3     | 37 $\pm$ 8.7     | 17 $\pm$ 11     | 180 $\pm$ 110    |
| Relative affinities (PG$_3$ vs. PG$_2$) | 7.3               | 3.8              | 4.8             | 3.5            | 78 |

* 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.

Effects of TXA$_3$ 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 hydroperoxide; $^{\Delta^{17}}$U46619 (1 $\mu$m) before addition of the diene or triene analogue (2 $\mu$m) or of collagen (2 $\mu$g/ml). SQ29548 completely blocked aggregation by either U46619 or $^{\Delta^{17}}$U46619 and inhibited collagen-induced aggregation by $\sim$50% (data not shown).

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 $\mu$m to measure the potency of each compound in platelet aggregation (Fig. 6). The threshold concentrations ranged from 0.5 to 0.8 $\mu$m for U46619 and from 0.7 to 1 $\mu$m 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 $\mu$m) before addition of the diene or triene analogue (2 $\mu$m) or of collagen (2 $\mu$g/ml). SQ29548 completely blocked aggregation by either U46619 or $^{\Delta^{17}}$U46619 and inhibited collagen-induced aggregation by $\sim$50% (data not shown).
human PRP, PGH₃ was found to isomerize to PGD₃ with a t₁/₂ of 90 s, unlike PGH₂ which has a t₁/₂ of 350 s. PGD₃ is also reported to be somewhat more potent at inhibiting aggregation than PGD₂ (70), both of which cause an increase in cyclic AMP. To block the putative inhibitory effects of any PGD₃, 1 μM adeny- late 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 TxA₂ reaction (Reaction 2).

Both PGH₂ and PGH₃ 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 TxA₂ or TxA₃ are approximately equipotent.

Comparison of PGI₂ and PGI₃ as Inhibitors of Platelet Aggregation—PGI₂ or PGI₃ (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 IC₅₀ values were 0.92 ± 0.28 nM and 1.30 ± 0.18 nM for PGI₂ and PGI₃, respectively. Thus, the potencies of PGI₂ and PGI₃ 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.

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 Ca²⁺ mobilization with the indicated concentrations of PGE₂, PGE₃, PGF₂α, or PGF₃α. 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.
Phospholipases—a cPLA2α is the key phospholipase involved in AA release in most PG forming cells (72). Previous studies have shown that cPLA2α 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 cPLA2α, DHA-containing phospholipids are essentially inactive with cPLA2α (76–78). Certain sPLA2 forms can also participate in PG biosynthesis (79, 80). Because of the nature of the interaction with its substrates, sPLA2 does not discriminate among 2-position acyl groups (81). In short, neither cPLA2 nor sPLA2 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 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 and 50% less AA to be oxygenated (92–94). A situation like this could occur in solution 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 sPLA2 is the operative phospholipase (95).

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 sPLA2 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
PGH2 and PGH3 (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 PGI2 versus PGI1 using human platelets.

The EP1, EP2, and EP3 receptors bound less well and were less responsive to PGE3 than PGE2. 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 (107). There are functions such as salt and water metabolism in the kidney that involve these two enzymes, and renal PGE2 synthesis is diminished with no detectable production of PGE3 in rats fed diets having elevated levels of fish oil (108).

PGIS and TXAS are reported to be similarly reactive with PGH2 and PGH3 (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.

FIGURE 6. Comparison of potencies of U46619 and 17U46619 for platelet aggregation. Human PRP (2.25 × 10^6 platelets per 0.4 ml) was treated with various concentrations of either U46619 or 17U46619 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 17U46619 was 1.2 times higher than for U46619 with platelets from each donor.
Ca2+ were probably compromised by the formation of PGD3 from PGH3 and PGD3 being a potent anti-aggregatory compound (70). In general, PGH3 appears to be significantly less stable than PGH2 in the aqueous systems used for our enzyme assays; PGH3 is rapidly converted to HHTE and malondialdehyde whereas spontaneous conversion of PGH2 to the homologous products is relatively slow.

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).

**Acknowledgments**—We thank Dr. Stephen C. Fischer and Dan Foster for guidance in making Ca2+/H11001 measurements and Dr. Nisha Palackal of Cayman Chemical Company for help with PGDS assays. We thank Dr. William E. M. Lands for his advice and encouragement during the course of these studies.

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