Coupling between Cyclooxygenase, Terminal Prostanoid Synthase, and Phospholipase A<sub>2</sub>*

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We have recently shown that two distinct prostaglandin (PG) E<sub>2</sub> synthases show preferential functional coupling with upstream cyclooxygenase (COX)-1 and COX-2 in PG<sub>E2</sub> biosynthesis. To investigate whether other lineage-specific PG synthases also show preferential coupling with either COX isozyme, we introduced these enzymes alone or in combination into 293 cells to reconstitute their functional interrelationship. As did the membrane-bound PG<sub>E2</sub> synthase, the perinuclear enzymes thromboxane synthase and PGI<sub>2</sub> synthase generated their respective products via COX-2 in preference to COX-1 in both the A23187-induced immediate and interleukin-1-induced delayed responses. Hematopoietic PGD<sub>2</sub> synthase preferentially used COX-1 and COX-2 in the A23187-induced immediate and interleukin-1-induced delayed PGD<sub>2</sub>-biosynthetic responses, respectively. This enzyme underwent stimulus-dependent translocation from the cytosol to perinuclear compartments, where COX-1 or COX-2 exists. COX selectivity of these lineage-specific PG synthases was also significantly affected by the concentrations of arachidonate, which was added exogenously to the cells or supplied endogenously by the action of cytosolic or secretory phospholipase A<sub>2</sub>. Collectively, the efficiency of coupling between COXs and specific PG synthases may be crucially influenced by their spatial and temporal compartmentalization and by the amount of arachidonate supplied by PL<sub>A2</sub>s at a moment when PG production takes place.

Biosynthesis of prostaglandins (PGs)<sup>1</sup> through the cyclooxygenase (COX) pathway involves oxidation and subsequent isomerization of membrane-derived arachidonic acid (AA) via three sequential enzymatic reactions. The initial step of this metabolic pathway is the stimulus-induced liberation of AA from membrane glycerophospholipids by the action of phospholipase A<sub>2</sub> (PL<sub>A2</sub>) enzymes, including cytosolic PL<sub>A2</sub><sub>a</sub> (cPL<sub>A2</sub>; group IVA) and several secretory PL<sub>A2</sub> (sPL<sub>A2</sub>) isoforms (groups IIa, IID, V, and X) (1–10). The released AA is sequentially metabolized to PGG<sub>2</sub> and then to PGH<sub>2</sub> by either COX-1 or COX-2. PGH<sub>2</sub> is then converted to various bioactive PGs (thromboxane (TX) A<sub>2</sub>, PGD<sub>2</sub>, PGE<sub>2</sub>, PGF<sub>2</sub>alpha, and PGI<sub>2</sub>) by the respective terminal PG synthases, which have different structures and exhibit cell- and tissue-specific distributions.

Segregated utilization of COX-1 and COX-2 in the PG bio-synthetic events has been demonstrated by a number of cellular biological, pharmacological, and genetic studies (3, 11–17). Generally, the constitutive COX-1 is mainly utilized in the immediate PG biosynthesis, which occurs within several minutes after stimulation with Ca<sup>2+</sup> mobilizers, whereas the inducible COX-2 is an absolute requirement for delayed PG biosynthesis, which lasts for several hours after proinflammatory stimuli. When cells are first treated with proinflammatory stimuli and subsequently exposed to Ca<sup>2+</sup> mobilizers, the induced COX-2 can also promote the immediate response (priming response) (3, 18–22). However, the precise molecular mechanisms underlying the functional segregation between the two COXs are still obscure. Although specific coupling between COXs and particular PL<sub>A2</sub> subtypes have been proposed (23, 24), subsequent coexpression studies have clearly demonstrated that both cPL<sub>A2</sub> and several sPL<sub>A2</sub> isoforms (groups IIa, IID, V, and X) are capable of supplying AA to both COX-1 and COX-2 in the immediate responses and mainly to COX-2 in the delayed responses (3–6). Moreover, studies using cPL<sub>A2</sub> null mice have provided unequivocal evidence that cPL<sub>A2</sub> is essential for both immediate and delayed phases of PG generation (25, 26). The actions of sPL<sub>A2</sub> isoforms in either phase appear to be cell type-specific and depend on their temporal expression, secretion process, and sorting into particular membrane microdomains (4, 6).

It has been suggested that the two COX isozymes are differently coupled with specific terminal PG synthases. For instance, rat peritoneal macrophages produce TXA<sub>2</sub> and PGD<sub>2</sub> through COX-1 in the A23187-induced immediate response and PGE<sub>2</sub> and PGI<sub>2</sub> through COX-2 in the lipopolysaccharide-induced delayed response (13, 18, 27). TXA<sub>2</sub> generation by activated platelets depends entirely on COX-1 (14), whereas PGE<sub>2</sub> forms in response to various stimuli is almost exclusively derived through COX-2, although COX-1 can participate in the formation of PGE<sub>2</sub> (16). The endoplasmic reticulum; HUVEC, human umbilical vein endothelial cells.

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§ The abbreviations used are: PG, prostaglandin; AA, arachidonic acid; COX, cyclooxygenase; PL<sub>A2</sub>, phospholipase A<sub>2</sub>; cPL<sub>A2</sub>, cytosolic PL<sub>A2</sub>; sPL<sub>A2</sub>, secretory PL<sub>A2</sub>; sPL<sub>A2</sub>-V, group V sPL<sub>A2</sub>; sPL<sub>A2</sub>-IID, group IID sPL<sub>A2</sub>; PGE<sub>2</sub>, prostaglandin E<sub>2</sub> synthase; cPGES, cytosolic PGES; mPGES, membrane-bound PGES; PGDS, PGD<sub>2</sub> synthase; HPGDS, hematopoietic PGD<sub>2</sub> synthase; PGIS, prostaglandin I<sub>2</sub> synthase; TX, thromboxane; TXS, TX synthase; FITC, fluorescein isocyanate; HEK, human embryonic kidney; IL-1, interleukin-1; PBS, phosphate-buffered saline; TBS-T, 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl and 0.1% Tween 20; FLAP, 5-lipoxygenase-activating protein; ER, endoplasmic reticulum; HUVEC, human umbilical vein endothelial cells.

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production by osteoblasts occurs predominantly through COX-2 irrespective of the co-presence of COX-1 (19, 22). In the biphasic production of PGD₂ by activated mouse cultured mast cells, only COX-1 is utilized in the immediate phase and only COX-2 in the delayed phase (12, 28). In a rat inflammatory model, COX-2-selective inhibitors reduce the accumulation of PGE₂ but not of other PGs (18). Furthermore, the different enzyme kinetics of each terminal synthase could create a situation in which the ratio of the PG products follows a more complex pattern (31).

Conversion of PGH₂ to TXA₂ and PGL₃ is catalyzed by TX synthase (TXX) and PGL synthase (PGIS), respectively, both of which belong to the cytochrome P-450 family and are reportedly localized in the endoplasmic reticulum (ER) and perinuclear membranes (32–34). PGG₂ synthase (PGDS), which isomerizes PGH₂ to PGG₂, occurs in two distinct forms, the lipocalin-type PGDS (L-PGDS), a secretory enzyme known as β-trace that is abundantly present in the central nervous system (35), and hematopoietic PGDS (H-PGDS), which represents the α class of the cytosolic glutathione-S-transferase family (36). The lung and liver types of PGF synthase are cytosolic proteins with high homology that belong to the aldo-keto reductase family (37, 38). Several proteins that exhibit PGE₂ synthase (PGES) activity have also been identified to date; they are the constitutive cytosolic PGES (cPGES), which is identical to p23 (39), the inducible, perinuclear membrane-bound PGES (mPGES), which was originally designated MGST1-L1 (for membrane-bound GST1-like-1) (40–42), and the cytosolic glutathione S-transferase isoforms μ2 and μ3 (43). The expression of mPGES is strongly induced by proinflammatory stimuli in vitro and at inflamed sites in vivo and is down-regulated by anti-inflammatory glucocorticoids (40, 42).

More recently, we have demonstrated by coexpression and antisense experiments that cPGES and mPGES favor COX-1 and COX-2, respectively, over the other for conversion of exogenous and endogenous AA to PGE₂ (39, 40). This finding has provided further support for the hypothesis that there is selective functional linkage between COX isozymes and terminal PG synthases. In this study, we have extended our coexpression approach to clarify diverse functional couplings between COXs and several other lineage-specific PG synthases (TXX, PGIS, and H-PGDS). Our results suggest that lineage-specific PG synthases are classified into three categories in terms of their localization and COX preference: (i) the perinuclear enzymes that prefer COX-2 (TXX, PGIS, and mPGES), (ii) the cytosolic enzyme that prefers COX-1 (cPGES), and (iii) the translocating enzyme that utilizes both COXs depending on the stimulus (H-PGDS). Alterations in AA supply through the stimulus approach to clarify diverse functional couplings between COXs and terminal PG synthases.

**EXPERIMENTAL PROCEDURES**

**Materials**—The goat anti-human COX-2 and rabbit anti-human cPLA₂ antibodies were purchased from Santa Cruz. The rabbit anti-human H-PGDS antibody was described previously (44). Rat TXX and mouse H-PGDS cDNAs were obtained by reverse-transcriptase polymerase chain reaction using mRNAs purified from rat platelets and mouse bone marrow-derived mast cells, respectively, as templates using 5’- and 3’-primers corresponding to the N- and C-terminal 23-base pair nucleotide sequences. The touchdown PCR condition was 94°C for 30 s and then 30 cycles of 94°C for 5 s and 68°C for 4 min with Advanta cDNA polymerase max (Clontech) using a DNA thermal cycler (PerkinElmer Life Sciences). Human endothelial cell-derived PGIS cDNA was derived previously (45). LipofectAMINE PLUS reagent, Opti-MEM medium, and TRIZol reagent were obtained from Life Technologies. Mouse anti-FLAG epitope monoclonal antibody was purchased from Sigma. Rabbit anti-human COX-1 antibody, AA, and the enzyme immunoassay kits for TXB₂ and 6-keto-PGF₁α were obtained from Cayman Chemical. Oligonucleotide primers were from Greiner.

Canetin, hygromycin, zeocin, and the mammalian expression vectors pcRS1.1, pCDNA3.1/His (+)

, and pCDNA3.1/zeo (+) were obtained from Invitrogen. A23187 was purchased from Calbiochem. Human interleukin-1β (IL-1) was purchased from Genzyme. Fluorescein isothiocyanate conjugated goat anti-mouse IgG, FITC-rabbit anti-goat IgG, FITC-goat anti-rabbit IgG antibodies, and horseradish peroxidase conjugated anti-rabbit and anti-goat antibodies were purchased from Zymed Laboratories Inc. Other reagents were obtained from Wako Pure Chemical Industries. Human embryonic kidney (HEK) 293 cells were cultured in RPMI 1640 medium containing 10% (v/v) fetal calf serum as described previously (2–6).

**Construction of FLAG-tagged TXX and PGIS cDNA**—To obtain C-terminally FLAG-epitope-tagged TXX, PCR was using TXX/ pcRS1.1 as a template with a primer 5′-TGA GTC ATC ATC GTC GTG TTA GTA GTC GGC TCA AAT CCT GAC 3′ (the FLAG epitope is underlined) in combination with a primer 5′-ATG GAA GTG TTG GGG CTT CTC 3′. To obtain C-terminally FLAG-tagged PGIS, PCR was performed with PGIS/pCDNA3.1/His (+) as a template with primers 5′-TATA CTT GTC ATC ATC GTC GTG TTA GTA GGC TCA AAT CCT GAC 3′ and 5′-ATG GCT TGG GGG CTC 3′. The PCR reaction was carried out with 25 cycles of denaturation for 5 s at 94°C and annealing and extension for 4 min at 68°C. The fragments obtained were each subcloned into the pcRS1.1 vector and sequenced.

**Establishment of Transfectants**—Establishment of HEK293 cells stably expressing either COX-1 or COX-2 and those coexpressing COX-1 and COX-2 and those expressing H-PGDS and TXS, was performed, respectively, using a PGD₂ radioimmunoassay kit (Amersham Pharmacia Biotech) as described previously (2–6). The cells were cloned by limiting dilution in the presence of 50 µg/ml zeocin. Expression of cPLA₂ was assessed by immunoblotting.

**Activation of 293 Cells**—All procedures were described in our previous reports (2–6). Briefly, HEK293 cells (5 × 10⁶/ml) were seeded into each well of 24- or 48-well plates in 1 ml and 0.5 ml of culture medium, respectively. After culturing for 4 days, the cells were washed once with culture medium and then incubated with 250 µl (24-well plate) or 100 µl (48-well plate) of various concentrations of AA or 10 µM A20187 in medium containing 1% fetal calf serum for 30 min (immediate response) or 1 ng/ml IL-1 in medium containing 10% fetal calf serum for 4 h (delayed response). PDG₂ released into the supernatants was quantified by a PGD₂ radioimmunoassay kit (Amersham Pharmacia Biotech) and TXB₂ and 6-keto-PGF₁α (stable endoproducts of TXA₂ and PGI₂, respectively) were quantified by their respective enzyme immunoassay kits (Cayman Chemicals).

To assess the transcellular action of sPLA₂ (3), cells expressing rat sPLA₂-V or mouse sPLA₂-III and those coexpressing COX-1 or COX-2 and those expressing H-PGDS (2.5 × 10⁶ cells/ml each) were cocultured for 4 days and then stimulated with 1 ng/ml IL-1 in medium containing 10% fetal calf serum for 4 h. TXB₂ and PDG₂ released into the supernatants were quantified.

Generation of these prostanoids was also verified by thin layer chromatography, as required for the experiments. Cells prelabelled with 24 h with 0.1 µCi/ml [³H]AA (Amersham Pharmacia Biotech) were treated with AA, A23187, or IL-1 as noted above, and the products were extracted with diethyl ether, methanol, 1M citric acid (30/4/1, v/v/v). The extracts were separated on silica gel 60 plates (Roche Molecular Biochemicals) with a solvent system of ether/petroleum ether/acetone (90/25/5, v/v/v). The radioactive products on the plate were detected by the BAS2000 imaging analyzer (Fuji). The Rf values of the products were compared with those of the authentic TXB₂, 6-keto-PGF₁α, and PGD₂ standards (Cayman Chemical).
antibodies against cPLA₂, COX-1, COX-2, H-PGDS, and FLAG epitope (1:5,000 dilution in TBS-T) for 2 h and then with horseradish peroxidase-conjugated second antibodies (1:5,000 dilution) for 1 h. After washing with TBS-T, protein bands were visualized with an enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech).

**RNA Blotting**—Approximately equal amounts (~10 μg) of the total RNAs obtained from the transfected cells were applied to separate lanes of 1.2% (w/v) formaldehyde-containing agarose gels, electrophoresed, and transferred to Immobilon-N membranes (Millipore). The resulting blots were then probed with the respective cDNA probes that had been labeled with [32P]dCTP (Amersham Pharmacia Biotech) by random priming (Takara Biomedicals). All hybridizations and membrane washing were carried out as described previously (2–6).

**Immunofluorescent Microscopic Analysis**—HEK293 cells transfected with COX-1, COX-2, FLAG-tagged TXS, FLAG-tagged PGIS, and H-PGDS were seeded onto collagen-coated cover glasses (Iwaki Glass) at 5 × 10⁴ cells/ml and cultured for 2 days. After removing the supernatants, the cells were fixed with 2% (w/v) paraformaldehyde in PBS for 30 min at 4 °C. Then the cells were incubated sequentially at room temperature in PBS containing 1% (w/v) bovine serum albumin (PBS-BSA) and 1% (w/v) saponin for 30 min, antibodies against COX-1, COX-2, FLAG epitope, or H-PGDS (1:500 dilution) in PBS-BSA, and FITC-conjugated second antibodies (1:100 dilution) in PBS-BSA. The coverslips were mounted on glass slides using Perma Fluor (Japan Tanner) and examined using a FLUOVIEW laser fluorescence microscope (Olympus).

**Measurement of PG Synthase Activity**—TXS, PGIS, and PGDS activities in cell lysates were measured by assessment of conversion of PGH₂ to TXB₂, 6-keto-PGF₁α, and PGD₂, respectively, as previously performed with PGES activity (39, 40). Briefly, the cells were scraped off from the dishes and disrupted by sonication using Branson Sonifier (10 s, 3 times, 1-min interval) in 400 μl of 10 mM Tris-HCl (pH 8.0). After centrifugation of the sonicates at 1,700 × g for 10 min at 4 °C, the supernatants were used as the enzyme source. An aliquot of each lysate (10 μg protein equivalents) was incubated with 0.5 μg of PGH₂ for 30 s at 24 °C in 0.1 ml of 1% Tris-HCl (pH 8.0) containing 1 mM GSH and 5 μg of indomethacin. After terminating the reaction by the addition of 100 μM FeCl₂, TXB₂, 6-keto-PGF₁α, and PGD₂ concentrations in the supernatants were quantified. Protein concentrations were determined by the protein assay kit (Pierce) using bovine serum albumin as a standard.

**Statistical Analysis**—Data were analyzed by Student’s t test. Results are expressed as the mean ± S.E., with p < 0.05 as the limit of significance.

### RESULTS

**Establishment of Transfectants**—To investigate the functional coupling between COXs and terminal PG synthases, cDNAs for TXS, PGIS, and H-PGDS were each transfected into COX-1- or COX-2-expressing 293 cells, which we have previously established (3). Expression levels of COXs and terminal synthases, as assessed by immunoblotting (for COX-1 and COX-2) or RNA blotting (for TXS, PGIS, and H-PGDS) in the transfectants obtained, are shown in Fig. 1A. Transcripts for TXS, PGIS, and H-PGDS were minimally detected in parental 293 cells, even after 33 cycles of amplification by reverse-transcriptase polymerase chain reaction using specific primers for human TXS, PGIS, and H-PGDS (data not shown).

We compared the expression levels of COXs and specific synthases in 293 transfectants with those in several normal cells (rat platelets, rat peritoneal macrophages, human umbilical vein endothelial cells (HUVEC), and rat mastocytoma RBL-2H3 cells), which produce TXA₂, PGI₂, and PGD₂, respectively, as previously described (6, 18, 64, 65). Expression of the two COXs (COX-1 and COX-2) and three terminal synthases (TXS, PGIS, and H-PGDS) overexpressed in 293 cells. B and C, comparison of COX (B) and terminal synthase (C) expression in 293 transfectants and several normal cells. The expression of COX-1 and -2 (A and B) was assessed by immunoblotting and that of TXS and PGIS (A and C) was assessed by RNA blotting. H-PGDS expression was assessed by RNA blotting (A) and immunoblotting (C). Comparable amounts of RNA (10 μg) and protein (50 μg) were applied to each lane in RNA blotting and immunoblotting, respectively. Equal loading of RNA in each lane was verified by reprobing the membranes with glyceraldehyde-3-phosphate dehydrogenase (data not shown). Preparation and culture of rat platelets, macrophages (with (+) or without (−) treatment with 5 μg/ml lipopolysaccharide), RBL-2H3 cells, and HUVEC were described previously (6, 18, 64, 65). D, measurement of in vitro COX synthase activities. Homogenates of 293 cells with (+) or without (−) transfection of TXS, PGIS, or H-PGDS were incubated with PGH₂, and the formation of respective products was quantified. RBL, RBL-2H3 cells.
Coupling between COX, Terminal Prostanoid Synthase, and PLA2

**Fig. 2. Conversion of exogenous AA to prostanoids.** The cells shown in Fig. 1A were incubated for 30 min with the indicated concentrations of AA, and TXB2 (A), 6-keto-PGF1α (B), and PGD2 (C) released into the supernatant were quantified as described under “Experimental Procedures.” 6-Keto-PGF1α formation by transfected PGIS, calculated by subtracting that produced by cells expressing either COX alone from that produced by COX/PGIS cotransfectants, is shown in the inset of panel B. Values are the mean ± S.E. of 3–5 experiments.

Expression level in 293 transfectants was similar to that in HUVEC and was significantly higher than that in macrophages (Fig. 1C). H-PGDS expression levels in 293 transfectants and in RBL-2H3 cells were comparable (Fig. 1C). Thus, the expression levels of COXs and specific synthases overexpressed in 293 cells were within physiological ranges.

To confirm that terminal synthases transfected into 293 cells were functionally active, we measured the in vitro activities of TXS, PGIS, and PGDS by incubating the homogenates of the respective transfectants with PGH2. As shown in Fig. 1D, the production of TXB2, 6-keto-PGF1α, and PGD2 was greatly elevated in cells transfected with TXS, PGIS, and H-PGDS, respectively, relative to control cells. Conversion of Exogenous AA—We next examined the conversion of exogenous AA to TXB2 (Fig. 2A), 6-keto-PGF1α (Fig. 2B), and PGD2 (Fig. 2C) to assess the functional coupling of TXS, PGIS, and H-PGDS, respectively, with COX-1 and COX-2 by incubating the intact cells for 30 min with various concentrations of AA.

Consistent with negligible expression of endogenous TXS (Fig. 1), parental cells and cells expressing COX-1 or COX-2 alone did not appreciably produce TXB2, even at the highest AA concentration (10 μM) (Fig. 2A). In cells coexpressing COX-1 and TXS, significant production of TXB2 became detectable at 2–5 μM AA and increased markedly at 5–10 μM (Fig. 2A, left). In cells coexpressing COX-2 and TXS, TXB2 generation was evident even at 1 μM AA and reached a maximal level with a plateau at 2–10 μM (Fig. 2A, right).

Irrespective of virtually no expression of PGIS (Fig. 1), small but significant increases in the accumulation of 6-keto-PGF1α, from exogenous AA were observed in both COX-1- and COX-2-expressing cells as compared with control cells (Fig. 2B). In cells expressing COX-1 alone, 6-keto-PGF1α formation became evident at 1–2 μM AA and increased almost linearly with increasing concentrations of AA (Fig. 2B, left). Cells expressing COX-2 alone also produced 6-keto-PGF1α, with a dose dependence similar to that observed in those expressing COX-1 alone (Fig. 2B, right), yet the amount of 6-keto-PGF1α produced by COX-2-expressing cells was considerably lower than that produced by COX-1-expressing cells. Cells cotransfected with COX-2 and PGIS produced 5-fold more 6-keto-PGF1α at all AA concentrations than did those expressing COX-2 alone (Fig. 2B, right). By contrast, conversion of AA to 6-keto-PGF1α by COX-1/PGIS cotransfectants was comparable with that by COX-1 single transfectants below 5 μM AA, and the increase became apparent only at 10 μM AA (Fig. 2B, left). When the amounts of 6-keto-PGF1α produced by cells expressing either COX alone were subtracted from those produced by the respective COX/PGIS double transfectants, the AA requirement profile for 6-keto-PGF1α formation after transfection of PGIS (Fig. 2B, inset) was similar to that of TXS (Fig. 2A).

Low but significant amounts of PGD2 were produced from exogenous AA in cells expressing COX-1 or COX-2 alone, and cotransfection of H-PGDS with either COX led to a marked increase in PGD2 formation at all AA concentrations tested (Fig. 2C). PGD2 production in COX-1/H-PGDS cotransfectants increased modestly at 2–5 μM AA and markedly at 5–10 μM AA (Fig. 2C, left). In COX-2/H-PGHS cotransfectants, PGD2 generation was detectable at 1 μM AA and increased almost linearly up to 10 μM AA without apparently reaching saturation (Fig. 2C, right).

Conversion of Endogenous AA—Metabolism of endogenous AA, which is supplied by cPLA2 intrinsically expressed in HEK293 cells at a low level (2, 3), to each PG was examined next by stimulating the cells with A23187 for 30 min (immediate response) (Fig. 3, left) or with IL-1 for 4 h (delayed response) (Fig. 3, right).

When COX-1/TXS- and COX-2/TXS-expressing cells were stimulated with A23187, TXB2 production increased 5- and 40-fold, respectively, relative to replicate control cells or cells expressing either COX alone, in which TXB2 generation was minimal (Fig. 3A, left). After IL-1 stimulation, TXB2 generation by COX-2/TXS-expressing cells increased markedly as compared with replicate control cells or cells expressing COX-2 alone, whereas coexpression of TXS with COX-1 led to a minimal increase in TXB2 generation (Fig. 3A, right). Thus, in this experimental setting, TXS is coupled with COX-2 in preference to COX-1 in both immediate and delayed responses.

When cells expressing COX-1 or COX-2 alone were stimulated with A23187, immediate 6-keto-PGF1α generation increased 10- and 2.5-fold, respectively, as compared with replicate control cells (Fig. 3B, left), suggesting COX-1 preference of the endogenous PGL2 biosynthetic activity. Further transfection of PGIS into COX-2-expressing cells led to a marked increase in A23187-induced 6-keto-PGF1α generation. In contrast, A23187-induced 6-keto-PGF1α formation by cells cotransfected with COX-1 and PGIS did not differ significantly.
from that by cells expressing COX-1 alone. IL-1-induced delayed 6-keto-PGF$_{1\alpha}$ generation was increased significantly only in cells cotransfected with COX-2 and PGIS (Fig. 3B, right). These results together with those of the experiments using exogenous AA (Fig. 2B) suggest that PGIS prefers COX-2 in marked preference to COX-1 in the 293 cell system.

An increase in 6-keto-PGF$_{1\alpha}$ formation in cells transfected with COX-1 alone (i.e. without transfection of PGIS) suggests that 293 cells endogenously express another type of PGIS that prefers COX-1. However, we cannot rule out the possibility that the product detected in the COX-1 transfectants is an immunoreactive substance related but not identical to 6-keto-PGF$_{1\alpha}$, even though it comigrated with the authentic 6-keto-PGF$_{1\alpha}$ standard on thin layer chromatography (data not shown). We did not focus on this putative COX-1-preferential PGIS further in this study because of the uncertainty of its molecular nature and of its small production.

After A23187 stimulation, cells expressing COX-1 or COX-2 alone produced substantial amounts of PGD$_2$, and COX-1-dependent production of PGD$_2$ was greatly augmented by co-transfection of H-PGDS (~7-fold increase) (Fig. 3C, left). Co-expression of COX-2 and H-PGDS resulted in only up to a 2-fold increase in A23187-induced PGD$_2$ generation as compared with COX-2 single transfectants (Fig. 3C, left). In contrast, IL-1-induced delayed PGD$_2$ generation, which increased mod-

**Fig. 3.** Metabolism of endogenous AA to prostanoids. The cells shown in Fig. 1A were incubated for 30 min with 10 µM A23187 (left) or for 4 h with 1 ng/ml IL-1 (right), and TXB$_2$ (A), 6-keto-PGF$_{1\alpha}$ (B), and PGD$_2$ (C) released into the supernatant were quantified. Values are the mean ± S.E. of 3–5 experiments.

**Fig. 4.** Effects of cPLA$_2$ cotransfection on the production of prostanoids. A–C, COX/terminal PG synthase double transfectants with (+) or without (−) further transfection of cPLA$_2$ were stimulated with A23187 for 30 min (left) or IL-1 for 4 h (right), and TXB$_2$ (A), 6-keto-PGF$_{1\alpha}$ (B), and PGD$_2$ (C) released into the supernatant were quantified. Values are the mean ± S.E. of 3–5 experiments. The expression of cPLA$_2$, as assessed by immunoblotting (10^5 cell equivalents/lane), is shown in the inset. D, comparison of the expression levels of cPLA$_2$ in 293 cells transfected with cPLA$_2$, rat platelets, and RBL-2H3 cells (RBL) as assessed by immunoblotting (50 µg of protein/lane).
estly in cells expressing COX-2 alone, was further increased 2-fold by cotransfection of H-PGDS, whereas no appreciable increase in delayed PGD2 generation occurred in COX-1/H-PGDS double transfectants (Fig. 3C, right). Thus, unlike TXS and PGIS, H-PGDS exhibits dual COX selectivity according to the stimulus such that COX-1 and COX-2 are preferentially utilized in the A23187-induced immediate and IL-1-induced delayed responses, respectively. This situation resembles that observed in mast cells (12).

Increased Supply of Endogenous AA by Overexpression of cPLA2—Considering that conversion of exogenous AA to each PG via COX-1 occurs only at higher AA concentrations (Fig. 2), we next aimed to examine how an increased supply of endogenous AA alters the profiles of coupling between COXs and PG synthases. To this end, we introduced cPLA2 into each of the double transfectants. The expression of cPLA2 in 293 cells, as assessed by immunoblotting, is shown in the inset of Fig. 4, A–C. As shown in Fig. 4D, cPLA2 expression level overexpressed in 293 cells was roughly comparable with that endogenously expressed in rat platelets and RBL-2H3 cells.

Supplying more endogenous AA by A23187-induced activation of overexpressed cPLA2 led to a more than 10-fold increase in COX-1/TXS-mediated TXB2 production, which reached a level comparable with that of COX-2/TXS cotransfectants (Fig. 4A, left). In contrast to the A23187-dependent event, IL-1-dependent TXB2 generation increased only minimally in cPLA2/COX-1/TXS triple transfectants (Fig. 4A, right). Transfection of cPLA2 into COX-2/TXS double transfectants increased TXB2 generation nearly 2-fold in both the A23187 (Fig. 4A, left)- and IL-1 (Fig. 4A, right)-dependent responses. These results are in line with the experiments using exogenous AA, in which COX-1/TXS coupling occurred only at higher AA concentrations, whereas TXB2 generation via COX-2 tended to become saturated as AA concentrations increased (Fig. 2A).

COX-2/PGIS-dependent immediate (Fig. 4B, left) and delayed (Fig. 4B, right) 6-keto-PGF1α generation increased severalfold after cotransfection of cPLA2. Unexpectedly, no significant augmentation of 6-keto-PGF1α generation was observed with either stimulus when cPLA2 was overexpressed in COX-1/PGIS-coexpressing cells (Fig. 4B). These results imply that even if the supply of endogenous AA by cPLA2 increases, PGIS appears to fail to metabolize COX-1-derived PGH2 to PGI2 in this setting.

Transfection of cPLA2 further augmented A23187-elicited immediate PGD2 generation, which increased ~4- and 2.5-fold in cPLA2/COX-1/H-PGDS and cPLA2/COX-2/H-PGDS triple transfectants, respectively, relative to the respective double transfectants (Fig. 4C, left). IL-1-induced delayed PGD2 generation increased up to 3-fold in COX-2/H-PGDS-expressing cells, whereas it increased only modestly in COX-1/H-PGDS-expressing cells, after cotransfection of cPLA2 (Fig. 4C, right). Thus, the phase-specific COX preference (COX-1 in the immediate response and COX-2 in the delayed response) is unaltered even...
when more endogenous AA is supplied by cPLA2.

Increased Supply of Endogenous AA by Transcellular Action of sPLA2—Several sPLA2s enhance AA metabolism in mammalian cells in autocrine, paracrine, and juxtacrine fashions (2-10, 21-24, 46). To assess whether or not AA supply by sPLA2 affects the coupling selectivity between COXs and terminal synthases in a manner different from that by cPLA2, we exploited the transcellular PG biosynthetic assay in which 293 cells expressing sPLA2 and those coexpressing COXs and terminal PG synthases were cocultured. In this system, sPLA2 secreted from the sPLA2-expressing cells acts on the neighboring COX-expressing cells and enhances PGE2 production by the latter (3).

When cells coexpressing COX-1/TXS or COX-2/TXS were cocultured with those expressing sPLA2-V and then stimulated with A23187, TXB2 generation increased ~4- and 2-fold, respectively, compared with their replicate cells cocultured with parental 293 cells (Fig. 5A, left). As a result, even though TXB2 generation still showed COX-2 preference, a significant level of TXB2 was also formed via sPLA2-V and COX-1. In the IL-1-dependent response, a significant increase in TXB2 generation by sPLA2-V was observed only in COX-2/TXS-coexpressing cells (Fig. 5A, right). Similar augmentation of TXB2 generation was observed when cells expressing sPLA2-IID were used in the transcellular assay (Fig. 5B). When the transcellular assay was conducted using COX-1/PGIS and COX-2/PGIS cotransfectants, augmentation of 6-keto-PGF1α formation by sPLA2-V was evident only in COX-2/PGIS-coexpressing cells (Fig. 5C). Furthermore, sPLA2-V preferentially increased PGD2 generation via COX-1/H-PGDS in the immediate response (Fig. 5D, left) and via COX-2/H-PGDS in the delayed response (Fig. 5D, right). Thus, the overall tendency of the augmentative effects of these sPLA2s is similar to that observed with the cPLA2 cotransfection study (Fig. 4).

Subcellular Distribution—Immunostaining of COX-1- and COX-2-transfected 293 cells with the respective anti-COX antibodies revealed that both COXs are mainly located in the perinuclear envelope (Fig. 6, A and B). In addition, the signal for COX-1 spread into the cytoplasm along the reticular compartment, indicating that it also resides in the ER membrane (Fig. 6A). These distributions of COXs are in agreement with several previous reports (3, 47, 48).

Localization of TXS and PGIS was assessed by transfecting the cells with FLAG-tagged TXS and PGIS, which were visualized by using an anti-FLAG epitope antibody. Specific binding of the anti-FLAG antibody to the epitope-tagged synthases was verified by immunoblotting, in which only a single band with the predicted molecular weight was detected in each of the transfectants (Fig. 7A). As shown in Fig. 6C, a strong TXS signal was detected in the perinuclear envelope as well as in the ER. In comparison with TXS (Fig. 6C), signal for PGIS was rather restricted to the perinuclear envelope and was barely detectable in the ER (Fig. 6D). Distribution of these enzymes was unchanged before and after cell activation (data not shown).

H-PGDS-transfected 293 cells were immunostained with an anti-H-PGDS antibody (Fig. 8). The specificity of the antibody was verified by immunoblotting, in which only a 26-kDa H-PGDS protein band appeared (Fig. 7B). In unstimulated cells, H-PGDS was distributed throughout the cytoplasm (Fig. 8A). In A23187-stimulated cells, the H-PGDS signal was detected in the intracellular compartment adjacent to the nucleus (Fig. 8B). After IL-1 stimulation, the H-PGDS signal largely overlapped with the nucleus, particularly with the perinuclear envelope (Fig. 8C). Recalling the distribution of the two COXs (Fig. 6, A and B), after A23187 stimulation H-PGDS translo-
cates into the intracellular domain where COX-1 is dominant, whereas after IL-1 stimulation it moves to the perinuclear region where both COX-1 and COX-2 exist.

**DISCUSSION**

It is now believed that segregated utilization of COX-1 and COX-2 occurs in the distinct PG biosynthetic pathways, even when they co-exist in the same cell. Although location of the two COXs in different subcellular compartments may be an explanation for this event (3, 47, 48), conflicting evidence has also been provided (49). In *vitro* enzymatic analyses have shown that effective catalysis by COX-2 can proceed at low substrate concentrations that are insufficient to sustain catalysis by COX-1 (50, 51). This finding is supported by subsequent cell biological studies, suggesting that COX-1 requires higher concentrations of the substrate than does COX-2 to exert its function (3, 39, 40, 52). However, the conclusion from these cellular studies has mostly been based on the production profiles of the major terminal product, PGE2, which is actually produced differently from either of the COX isozymes in several cell types (13, 18, 27), it is now important to clarify whether selective coupling between COXs and terminal synthases indeed occurs and, if so, how this selectivity is regulated.

To better address this issue, we have performed gain-of-function analysis by stably transfecting HEK293 cells with various PG-biosynthetic enzymes alone or in combination. These reconstitution studies have successfully demonstrated the functional linkage between various PLAs and two COXs (3–6) and between two COXs and two PGESs (39, 40). Although overexpression studies do not always reflect the true functions of endogenous proteins, they are one of the best recourses open to us in view of the nature of inhibitors, antagonists, activators, and antibodies used in previous studies. Note that the expression levels of cPLA2, COXs, and terminal synthases overexpressed in 293 cells in this study are within the ranges of those expressed in several normal cells (Figs. 1 and 4D). Moreover, although cell- and tissue-specific expression of various terminal PG synthases makes it difficult to directly compare their functional aspects in different cell lines, the present approach has enabled us to do so in a single type of cell. Our present studies on the three terminal synthases (TXS, PGIS, and H-PGDS) as well as previous reports on the two PGESs (39, 40) have revealed the occurrence of selective coupling between COXs and these enzymes (summarized in Table I) and have reinforced that the several mechanisms proposed to date, including enzyme compartmentalization (3, 47, 48), substrate availability (3, 39, 40, 52), and kinetic properties (31), can all influence the efficiency of coupling leading to the generation of each terminal product.

Our results indicate that TXS, which resides in the ER and perinuclear membranes (Fig. 6C), preferentially utilize COX-2 over COX-1 when limited amounts of AA are supplied and can efficiently metabolize COX-1-derived PGH2 when its supply is increased (Figs. 2–5). It is therefore likely that the coupling of TXS to COX is primarily dependent on the relative amount of PLAs/TX/PGS and, thus, is substrate-dependent. COX-1 is functional in the immediate TXB2 generation, during which high levels of AA are released in a short periods by a burst activation of PLAs, whereas COX-2 is a prerequisite for the delayed TXB2 generation, in which small amounts of AA are gradually supplied over a long period. Our reconstitution system agrees with the fact that TXA2 generation via COX-1 occurs in platelets, which express cPLA2 at a high level (14). Overall, the COX-2 selectivity of TXS appears to resemble that of mPGES, another perinuclear terminal synthase (40).

PGIS also utilizes COX-2 in marked preference to COX-1 (Figs. 2–5). This result is compatible with the findings that PGIL1 is preferentially produced via COX-2 in macrophages (27) and that COX-2 is a major source of systemic PGIL1 biosynthesis in healthy humans as demonstrated by a study using celecoxib, a COX-2-selective inhibitor (53). Of note, however, COX-2 preference of PGIS appears more strict than that of TXS and mPGES in our experimental setting (Figs. 2–5). Since the expression level of PGIS in 293 transfectants reached a level comparable with that expressed in HUVEC (Fig. 1C), it is unlikely that uncoupling of PGIS with COX-1 is due to its limited expression in the transfectants. Paradoxically, Wu and co-workers (48) show that PGIS and COX-1 can obviously couple in their settings. These seemingly discrepant observations can be reconciled if subcellular localization of these enzymes is taken into account (see below).

The concept that AA concentration is not the sole factor that could influence COX selectivity arises from the observation that H-PGDS, a cytosolic enzyme, exhibits unique COX coupling in that it prefers COX-1 and COX-2 in the immediate and delayed responses, respectively (Figs. 3–5). Irrespective of this fact, H-PGDS is preferentially linked with COX-2 when lower concentrations of AA are added exogenously (Fig. 2C), the event resembling that observed with TXS, PGIS (Fig. 2, A and B), and mPGES (40). Thus, the COX-1 preference of H-PGDS in A23187-stimulated cells cannot be simply explained by the AA concentration rule noted above.

Our finding that H-PGDS changes its subcellular location during cell activation (Fig. 8) sheds light on the regulatory mechanism for the segregated coupling of this enzyme with COXs. In our immunocytochemical studies, both COX-1 and COX-2 are localized in the perinuclear envelope, and only COX-1 is dispersed into the cytoplasm along the ER membrane (Fig. 6, A and B). TXS (Fig. 6C) and mPGES (40), which are preferentially linked with COX-2 but are still capable of utilizing COX-1 when combined with signaling PLAs, are located in the perinuclear envelope and ER. PGIS, which shows rather strict dependence on COX-2, resides predominantly in the perinuclear envelope (Fig. 6D). H-PGDS, which shows dual COX selectivity depending upon the phase of cell activation (Figs. 3C, 4C, and 5D), translocates...
into the perinuclear area outside the nuclear envelope (probably a part of the ER) after stimulation by A23187 and into the perinuclear envelope after stimulation by IL-1 (Fig. 8). As shown previously (39), PGES, which is strictly linked with COX-1, stays in the cytosol before and after cell activation.

Taking these observations together, we speculate that terminal PG synthases colocalized with both COXs in the perinuclear envelope are preferentially linked with COX-2, whereas those residing outside the perinuclear envelope (ER and cytosol) are selectively linked with the COX-1 that is distributed in the ER. In this scenario, AA may be presented to COX-2 in preference to COX-1 and in turn to terminal PG synthases in the perinuclear microenvironments, possibly due to the difference in enzymatic properties of the two COXs (3, 50–52) or to the presence of some putative perinuclear cofactor(s) that assists the selective transfer of AA to COX-2. The latter possibility is reminiscent of the role of FLAP (for 5-lipoxygenase-activating protein), which plays a pivotal role in presenting AA to 5-lipoxygenase around the perinuclear area in the leukotriene biosynthetic pathway (54). The AA accumulated around the perinuclear microenvironments, possibly due to the difference in enzymatic properties of the two COXs but rather appear to reflect those of the terminal PG synthases themselves. This situation is plausible, since each PG synthase has different $K_a$ and $V_{max}$ values for PGH$_2$. This implies that the ratios of the terminal products can vary according to the amounts of PGH$_2$ produced as a result of PLA$_2$/COX coupling at the moment when PG generation takes place.

Collectively, our studies provide additional evidence for the nature of the regulatory mechanisms of functional coupling between COXs and terminal PG synthases. The amounts of AA released by cPLA$_2$ or sPLA$_2$, the subcellular localization of COXs and terminal PG synthases, and the properties of the terminal PG synthases themselves all significantly affect the coupling efficiency between the enzymes in the biosynthetic pathway. The COX-1 versus COX-2 pathways can also be differently affected by other cellular factors, such as nitric oxide (62, 63).

Whether a certain FLAP-like cofactor(s) that might facilitate selective linkage between each COX and terminal PG synthase under particular conditions exists in cells remains to be elucidated.

Compartmentalization has now become a central dogma for the regulation of eicosanoid biosynthesis. cPLA$_2$ (55, 56) and ePLA$_2$s, including envelope. To which terminal PG synthases with cytosolic origin may be to the ER may be metabolized to PGs via the ER-localized COX-1, ene biosynthetic pathway (54). The AA accumulated around 5-lipoxygenase (57, 58) undergo translocation from the cytosol to 5-lipoxygenase around the perinuclear area in the leukotriene biosynthetic pathway (54). The AA accumulated around the perinuclear microenvironments, possibly due to the difference in enzymatic properties of the two COXs (3, 50–52) or to the presence of some putative perinuclear cofactor(s) that assists the selective transfer of AA to COX-2. The latter possibility is reminiscent of the role of FLAP (for 5-lipoxygenase-activating protein), which plays a pivotal role in presenting AA to 5-lipoxygenase around the perinuclear area in the leukotriene biosynthetic pathway (54). The AA accumulated around the perinuclear microenvironments, possibly due to the difference in enzymatic properties of the two COXs but rather appear to reflect those of the terminal PG synthases themselves. This situation is plausible, since each PG synthase has different $K_a$ and $V_{max}$ values for PGH$_2$. This implies that the ratios of the terminal products can vary according to the amounts of PGH$_2$ produced as a result of PLA$_2$/COX coupling at the moment when PG generation takes place.

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Coupling between COX, Terminal Prostanoid Synthase, and PLA₂

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Coupling between Cyclooxygenase, Terminal Prostanoid Synthase, and Phospholipase A_2
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