Engineering of a novel hybrid enzyme: an anti-inflammatory drug target with triple catalytic activities directly converting arachidonic acid into the inflammatory prostaglandin E₂

Ke-He Ruan¹, Vanessa Cervantes and Shui-Ping So

Department of Pharmacological and Pharmaceutical Sciences, The Center for Experimental Therapeutics and Pharmacoinformatics, University of Houston, College of Pharmacy, Houston, TX 77030, USA

¹To whom correspondence should be addressed.

E-mail: khruan@uh.edu

Cyclooxygenase isoform-2 (COX-2) and microsomal prostaglandin E₂ synthase-1 (mPGES-1) are inducible enzymes that become up-regulated in inflammation and some cancers. It has been demonstrated that their coupling reaction of converting arachidonic acid (AA) into prostaglandin (PG) E₂ (PGE₂) is responsible for inflammation and cancers. Understanding their coupling reactions at the molecular and cellular levels is a key step toward uncovering the pathological processes in inflammation. In this paper, we describe a structure-based enzyme engineering which produced a novel hybrid enzyme that mimics the coupling reactions of the inducible COX-2 and mPGES-1 in the native ER membrane. Based on the hypothesized membrane topologies and structures, the C-terminus of COX-2 was linked to the N-terminus of mPGES-1 through a transmembrane linker to form a hybrid enzyme, COX-2-10aa-mPGES-1. The engineered hybrid enzyme expressed in HEK293 cells exhibited strong triple-catalytic functions in the continuous conversion of AA into PGG₂ (catalytic-step 1), PGH₂ (catalytic-step 2) and PGE₂ (catalytic-step 3), a pro-inflammatory mediator. In addition, the hybrid enzyme was also able to directly convert dihomo-gamma-linolenic acid (DGLA) into PGG₁, PGH₁ and then PGE₁ (an anti-inflammatory mediator). The hybrid enzyme retained similar Kₘ and Vₘₐₓ values to that of the parent enzymes, suggesting that the configuration between COX-2 and mPGES-1 (through the transmembrane domain) could mimic the native conformation and membrane topologies of COX-2 and mPGES-1 in the cells. The results indicated that the quick coupling reaction between the native COX-2 and mPGES-1 (in converting AA into PGE₂) occurred in a way so that both enzymes are localized near each other in a face-to-face orientation, where the COX-2 C-terminus faces the mPGES-1 N-terminus in the ER membrane. The COX-2-10aa-mPGES-1 hybrid enzyme engineering may be a novel approach in creating inflammation cell and animal models, which are particularly valuable targets for the next generation of NSAID screening.

Keywords: cyclooxygenase (COX)/inflammation/prostaglandin E₂ (PGE₂)/prostaglandin E₂ synthase (PGES)/protein engineering

Introduction

In physiological conditions, endogenous prostaglandin E₂ (PGE₂) plays important roles in stem cell proliferation, tissue regeneration, wound repair, bone formation and other cell-developing functions (Murakami et al., 2002). PGE₂ deficiency, caused by certain non-steroidal anti-inflammatory drugs (NSAIDs), could mediate stomach ulcers and possibly impair stem cell development (North et al., 2007). However, in pathological conditions, PGE₂ has the tendency to be a pro-inflammatory and cancermediator (Murakami and Kudo, 2006). On the other hand, prostaglandin E₁ (PGE₁) is an important endogenous anti-inflammatory mediator and vasodilator. Endogenous PGE₁ and PGE₂ from dihomo-gamma-linolenic acid (DGLA) and arachidonic acid (AA) metabolisms, respectively, require two enzymes [cyclooxygenase (COX) and prostaglandin E synthase (PGES)] (Ruan, 2004; Ruan and Dogné, 2006). However, DGLA and AA also serve as common substrates for other prostanoids which perform diverse and opposite biological functions (Ruan, 2004; Ruan and Dogné, 2006). Synthesis of the specific endogenous prostanoids in the cells were generally uncontrollable until the recent discovery in which an engineered hybrid enzyme [‘Tri-Cat enzyme’, COX isoform-2 (COX-2) or isoform-1 (COX-1) linked to PGIS; (Fig. 1A)] demonstrated that the AA could be specifically converted into prostacyclin or prostaglandin I₂ (PGI₂) in the cells transfected with the cDNA of the Tri-Cat Enzyme (Ruan et al., 2006, 2008a, 2008b). Furthermore, this finding indicated that it is feasible to re-direct and control the COX pathway-mediated AA and other lipids' metabolisms in cells. However, a single design of the Tri-Cat enzyme which specifically directs the metabolism of AA into PGI₂ may not accurately represent other prostanoids’ syntheses mediated by different COX downstream enzymes. For example, microsomal prostaglandin E₂ synthase-1 (mPGES-1) (molecular mass 17 kDa) belongs to the glutathione family of enzymes, which is different from that of prostacyclin synthase (PGIS), a microsomal P450 enzyme with a 60 kDa molecular mass. In addition, instead of a single major membrane anchor domain at the N-terminal segment for PGIS, mPGES-1 has been proposed to have four transmembrane (TM) domains which span the ER membrane (Fig. 1B). Therefore, it becomes important to test how the specific PGE₁ and PGE₂ biosyntheses could be controlled and even re-directed by a similar engineering to that of the hybrid enzyme of COX linked to PGIS. In this paper, we have engineered a novel hybrid enzyme that links human COX-2 and mPGES-1 through a well-defined TM domain to form a novel Tri-Cat Enzyme, COX-2-10aa-mPGES-1. Characterization of the COX-2-10aa-mPGES-1 has revealed that the hybrid enzyme...
was able to integrate the triple catalytic functions of COX-2 and mPGES-1. The cells transfected with the cDNA of COX-2-10aa-mPGES-1 demonstrated the specific metabolism of DGLA and AA into PGE1 and PGE2, respectively. This has led us to further conclude that the biosynthesis of prostanoids through the COX pathway could be re-directed and controlled by the Tri-Cat enzyme engineering and gene-transferring, which could potentially lead to the correction of diseases caused by the unbalanced production of prostanoids.

Material and methods

Materials

HEK293 cell lines were purchased from ATCC (Manassas, VA). Medium for culturing the cell lines was purchased from Invitrogen. [3H]-PGH2 was purchased from Cayman Chemical Co. (Ann Arbor, MI). The following were purchased from Sigma (St Louis, MO): [14C]-AA, goat antirabbit IgG-FITC conjugate, saponin, streptolysin O, Triton X-100 and triethylenediamine. Mowiol 4-88 was purchased from Calbiochem (San Diego, CA).

Engineered cDNA plasmids with single genes encoding the COX-2 or mPGES-1 sequences The cDNAs of the COX-2 and mPGES-1 were cloned into the pcDNA 3.1 vector containing a cytomegalovirus early promoter using a polymerase chain reaction (PCR) cloning approach. The correct sequences were confirmed by DNA sequencing and endonuclease digestion analyses. The COX-2 was linked to mPGES-1 through the 10 amino acid (aa) sequence on the pcDNA 3.1(+) vector. This method was prepared by a PCR approach following the subcloning procedures provided by the vector company (Invitrogen). Briefly, the PGIS cDNA was excised from a plasmid of COX-2-10aa-PGIS in the pcDNA3.1(+) vector (Ruan et al., 2006) with Bam HI cutting sites at both ends (of the PGIS cDNA). The cDNA sequence of human mPGES-1 was taken from the pET27 vector with Bam HI cutting sites added to both ends using the PCR approach, and then substituted by ligation into the COX-2-10aa cDNA in the pcDNA 3.1(+) vector to complete the COX-2-10aa-mPGES-1 cDNA.

Expression of the syntheses in HEK293 cells The recombinant syntheses were expressed in HEK293 cells as described (Deng et al., 2002, 2003; Ruan et al., 2005). Briefly, the cells were grown and transfected with the purified cDNA of the recombinant protein by the Lipofectamine 2000 method (Hatae et al., 2002), following the manufacturer's instructions (Invitrogen). For the transient expression, ~48 h after transfection, the cells were harvested for further enzyme assays and a western blot analysis was performed. For the stable expression, the transfected cells were incubated with culture medium containing geneticin (G418) for several months. The cells stably expressing the recombinant syntheses were identified by enzyme assays and western blot analyses.

Cell culture HEK293 cells were cultured in a 100-mm cell culture dish with high glucose Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 1% antibiotic and antimycotic acid, and were grown at 37 °C in a humidified 5% CO2 incubator.

Electrophoresis and western blot The transfected or untransfected HEK293 cells were washed with ice-cold PBS (0.01 M phosphate buffer, pH 7.4, containing 0.15 M NaCl), and collected by centrifugation. After washing three times, the pellet was re-suspended in a small volume of the same buffer and stored in −80 °C, until further assays were performed. The membrane proteins were extracted by 10% SDS, separated by 7–12% (w/v)-PAGE under denaturing conditions and then transferred to a nitrocellulose membrane. Bands recognized by particular primary antibodies were visualized with horseradish peroxidase-conjugated secondary antibody and chromogenic peroxidase substrates (Lin et al., 2000). Normalization of the mPGES-1 in microsomal and detergent-solubilized proteins was based on the intensities of the bands in the immunoblot.

Enzyme activity determination for COX-2 and mPGES-1 using the high performance liquid chromatography (HPLC) method To determine the activities of the syntheses that converted AA into PGE2 or DGLA into PGE1 through the tri-catalytic functions, different concentrations of [14C]-AA or [14C]-DGLA were added to the harvested cells in a total reaction volume of 0.225 ml (25 μl cells + 200 μl 2.5 mM glutathione in PBS). After 0.5–5 min of incubation, the reaction was stopped by adding 0.2 ml 0.1% TFA (trifluoroacetic acid) and 0.2 ml 2 mM SnCl2. The mixture was briefly vortexed for 1 min to ensure termination of the reaction. After centrifugation (6000 rpm, 5 min), the supernatant was passed through a C18 octadeyl column (Honeywell Burdick & Jackson) and eluted with 0.5 ml acetone, which was then evaporated under a gentle stream of air. A solvent containing 0.1% acetic acid and 30% acetonitrile (solvent A) was used to re-dissolve the protein and load the sample onto an HPLC C18 column (Varian Microsorb-MV 100-5, 4.6 × 250 mm) using the solvent A with a gradient from 35 to 100% acetonitrile for 45 min at a flow rate of 1.0 ml/min. The metabolized [14C]-AA and [14C]-DGLA in the end-product profile...
were monitored directly by a flow scintillation analyzer (Packard 150TR).

**Enzyme activity determination for COX and PGES using enzyme immunoassay (EIA)** The supernatant of the reaction mixture as described earlier was diluted 100 times using PBS containing 0.1% bovine serum albumin and then used for the determination of the end-product, PGE₂, using an enzyme immunoassay (EIA) kit following the manufacturer’s instructions (Cayman Chemical Co.).

**Immunofluorescence staining** Transfected cells grown on a cover glass were washed with PBS and then incubated with 3.7% formaldehyde for 10 min. The cells were then blocked for 20 min before being incubated with 1% saponin for 20 min. This was followed by the addition of the primary antibody (10 µg/ml of affinity-purified anti-human COX-2 or mPGES-1 antibody) in the presence of 0.25% saponin and 10% goat serum for 1 h. After washing with PBS, the cells were incubated with the secondary antibodies (Lin et al., 2000; Deng et al., 2002) for another hour. Cells stained with the FITC- or Rhodamine-labeled antibody were viewed under the Zeiss Axioplan 2 epifluorescence microscope.

**Results**

**Design of a recombinant protein with triple catalytic activities directly converting DGLA and AA into PGE₁ and PGE₂.**

The previously engineered active hybrid enzyme, which linked human COX-2 to PGIS, was based on the topological arrangements using the proximity between the C-terminus of COX-2 (located on the ER lumen) and the N-terminus of the P₄50 enzyme, PGIS (located inside the ER membrane) (Fig. 1A). The approach of inventing a linkage from the C-terminus of COX-2 to the N-terminus of PGIS through 10-amino acid residues (10aa) (Trip-cat enzyme, Fig. 1A) could specifically produce PGI₂ from AA. This implies that other prostanoid biosyntheses could also be redirected by engineering different Tri-cat enzymes linking COX to different downstream synthases. To test this hypothesis, mPGES-1, with its four proposed TM domains (Fig. 1B, Jakobsson et al., 1999; Jegerschöld et al., 2008)) and with a different protein sequence and membrane topology than that of PGIS (Fig. 1) was selected for the engineering of a new Tri-cat enzyme [COX-2-10aa-mPGES-1 (Fig. 1B)] that may be able to directly convert AA or DGLA into PGE₂ or PGE₁, respectively. A possible explanation for this mechanism is that the movement of the first enzyme product is facilitated by the hydrophobic environment of the ER membrane. The engineered active fusion enzyme has provided a basis to help further understand the catalytic coordination between the COX and mPGES in the ER membrane using biophysics approaches.

**Expression of the engineered COX-2-10aa-mPGES-1 in an HEK cell line**

The expression of the recombinant COX-2-10aa-mPGES-1 protein was tested in an HEK293 cell line by a transient protein expression approach using Lipofectamine 2000 (Invitrogen). Forty-eight hours post-transfection (with COX-2-10aa-mPGES-1 cDNA), the cells expressing the COX-2-10aa-mPGES-1 were confirmed by western blot analysis using anti-COX-2 and mPGES-1 antibodies. We have also stained the fusion enzyme using the separate COX-2 and mPGES-1 antibodies, which showed a very similar staining to that of the mixed antibodies (data not shown). The expressed protein was identified by both antibodies, showing a correct molecular mass of ~91 kDa (Fig. 2, lane 1). In contrast, HEK293 cells co-transfected with equal amounts of the COX-2 and mPGES-1 enzymes produced a signal at their designated locations of 72 and 17 kDa, respectively (Fig. 2, lane 2). However, the HEK293 cells transfected with the vector pcDNA 3.1(+) (negative control, lane 3) did not express COX-2 or mPGES-1. To test whether the expressed COX-2-10aa-mPGES-1 will anchor to the ER membrane and adopt similar membrane topologies as that of the wild-type COX-2 and mPGES-1, fluorescence immunostaining was used for localizing the sub-cellular distribution of the engineered enzymes overexpressed in HEK293 cells. A similar ER staining pattern was clearly observed in the HEK293 cells expressing the engineered COX-2-10aa-mPGES-1 protein (Fig. 3) compared with the cells expressing individual COX-2 and mPGES-1 using either anti-human COX-2 (Fig. 3A) or anti-human mPGES-1 (Fig. 3B) antibodies. The results indicated that the engineered COX-2-10aa-mPGES-1 has similar ER membrane anchoring functions as the wild-type COX-2 and mPGES-1 enzymes. The ER membrane is a continuation of the nuclear membrane and is distributed through the cell. The fluorescence signal from the stained COX-2 and mPGES-1 proteins further implies that the proteins are localized in the ER membrane.

**Fig. 2.** Western blot analysis of the over-expressed recombinant proteins in HEK293 cells. The cells were grown for 24 h on 100 mm culture dishes until they were about 95% confluent and then transfected with the purified cDNA plasmid (pcDNA3.1(+)) using Lipofectamine 2000 following the manufacturer’s instructions (Invitrogen). For the co-transfection (lane 2), the cells were transfected with 12 µg of human COX-2 cDNA plasmid and 12 µg of human mPGES-1 cDNA plasmid. For the COX-2-10aa-mPGES-1 (lane 1) and pcDNA 3.1(+) (negative control, lane 3), 24 µg of each plasmid were used for each individual transfection. Approximately 48 h after transfection, the cells were harvested and used for western blot analysis. Equal amounts of protein (25 µg) were applied on a 7% SDS–polyacrylamide gel. Following electrophoresis, the protein was transferred to a nitrocellulose membrane which was probed with a mixture of rabbit anti-mPGES-1 and anti-COX-2 antibodies using a 1:500 dilution (Cayman, Ann Arbor, MI) and then stained with horseradish peroxidase-labeled goat-anti rabbit antibody using a Chemiluminescence kit (Amersham, England, UK). The numbers on the left represent the molecular mass (in kDa) of the proteins described.
catalytic steps, including the conversion of [14C]-AA into sing the COX-2-10aa-mPGES-1, which required the triple specifically converted into [14C]-PGE2 by the cells expres-

peptide antibody (A), and then incubated with the affinity-purified rabbit anti-COX-2 pcDNA vector (5) and (6). The cells were generally permeabilized by saponin, and then incubated with the affinity-purified rabbit anti-COX-2 peptide antibody (A) and mouse anti-mPGES antibody (B). The bound antibodies were stained by FITC-labeled goat-anti rabbit IgG (A) or Rhodamine-labeled goat anti-mouse IgG (B). The stained cells were examined by fluorescence microscopy.

**Directly converting AA into PGE2 by the engineered COX-2-10aa-mPGES-1 with triple catalytic activities through metabolic profile analysis**

Evidence for the conversion of AA into PGE2 by the single protein, COX-2-10aa-mPGES-1, was obtained analyzing the metabolic profile of the [14C]-AA using an HPLC assay. Figure 4A showed that the majority of the [14C]-AA was specifically converted into [14C]-PGE2 by the cells expressing the COX-2-10aa-mPGES-1, which required the triple catalytic steps, including the conversion of [14C]-AA into [14C]-PGG2, [14C]-PGH2 and finally into [14C]-PGE2. More interestingly, the [14C]-AA that was metabolized into [14C]-PGE2 by the triple catalytic reactions of the COX-2-10aa-mPGES-1 was more effective with less side products (less background, Fig. 4A) compared with that of the cells co-expressing the individual wild-type COX-2 and mPGES-1 (Fig. 4B). In other words, COX-2-10aa-mPGES-1 produced more PGE2 than that of the two individual parent enzymes, COX-2 and mPGES-1. Thus, the COX-2-10aa-mPGES-1 indeed exhibited effective triple catalytic activities in the direct conversion of AA to PGE2. In contrast, the cells expressing individual COX-2 (Fig. 4C) or mPGES-1 (Fig. 4D) failed to convert the [14C]-AA specifically into [14C]-PGE2. It shall be indicated that a small amount of [14C]-AA was converted into [14C]-PGE2 by the cells expressing COX-2 alone. However, it was anticipated that this could occur since COX-2 can convert [14C]-AA into [14C]-PGH2, which could be partially degraded into PGE2 by non-enzymatic reactions. Additional controls, including the cells transfected with pcDNA3.1 vector alone (Fig. 4E), and without gene transfection (Fig. 4F) failed to convert [14C]-AA into [14C]-PGE2. These negative controls further confirmed that the conversion of the [14C]-AA into [14C]-PGE2 by the cells expressing COX-2-10aa-mPGES-1 (Fig. 4A) was a result of its specific triple catalytic functions.

**Identification of the produced PGE2 using enzyme immunoassay (EIA)**

To further confirm that AA was converted into PGE2 by the cells expressing COX-2-10aa-mPGES-1, a specific antibody that recognizes PGE2 was used to identify the immuno-reactive property of the product from the enzyme assay. Figure 5 showed that an EIA was able to detect the PGE2 standard (Fig. 5D) and the product generated by the HEK293 cells expressing the COX-2-10aa-mPGES-1 single protein (Fig. 5A), or the co-expressed COX-2 and mPGES-1 (Fig. 5B) using the unlabeled AA as a substrate. In contrast, no PGE2 was detectable when untransfected HEK293 cells were used (Fig. 5C). In addition, the cells expressing COX-2-10aa-mPGES-1 were able to convert more AA into PGE2 than the cells co-expressing COX-2 and mPGES-1 (Fig. 5), which was identical to that of the metabolic profile analysis using [14C]-AA as a substrate, as described in Fig. 4. It should be mentioned that the increased activity was likely the result from the normalized expression levels of the fusion enzyme compared to the co-expressed COX-2 and mPGES-1 using western blot analysis.

**Directly converting dihomo-gamma-linolenic acid (DGLA) into PGE1, by the engineered COX-2-10aa-mPGES-1 with triple catalytic activities**

Another endogenous polyunsaturated fatty acid, DGLA (20:3 n=6), is also a substrate for COX-2-10aa-mPGES-1 and yields PGE1, which has been identified as an important endogenous and exogenous molecule with anti-inflammatory properties. PGE1 has been shown to inhibit human platelet aggregation in vitro, and can be a potent anti-atherosclerotic agent. Evidence for the conversion of DGLA into PGE1 by the single protein, COX-2-10aa-mPGES-1, was also given by HPLC analysis (Fig. 6A). The PGE1 produced was further identified by a standard (Fig. 6B). Thus, the COX-2-10aa-mPGES-1 indeed exhibited tri-catalytic activities in the direct conversion of DGLA into PGE1.

**Enzyme kinetics of the COX-2-10aa-mPGES-1 expressed in HEK293 cells**

A time course experiment for the direct conversion of AA into PGE2 by the cells expressing COX-2-10aa-mPGES-1 was compared with that of the cells co-expressing COX-2 and mPGES-1 (Fig. 7A). The COX-2-10aa-mPGES-1 displayed a slightly faster reaction kinetic, within the first 30 s, compared with that of the co-existing individual COX-2 and
mPGES-1 (Fig. 7A). However, non-significant differences were observed for the kinetic reaction at 60, 120 and 300 s between the two reaction systems (Fig. 7A). The COX-2-10aa-mPGES-1 Km values for the direct conversion of AA into PGI2 is approximately 0.5 \( \mu M \) (Fig. 7B), which is identical to that of the cells co-expressing the wild-type COX-2 and mPGES-1 (Fig. 7B). Surprisingly, the Km value of the COX-2 and mPGES-1 coupling reaction is almost one order less than that of the coupling reaction of COX-2 and PGIS with a Km value of 3.2 \( \mu M \) (Ruan et al., 2006).

Using the COX-2-10aa-mPGES-1 hybrid enzymes as an inflammatory target

The PGE2 produced by the hybrid enzyme is a pro-inflammatory mediator. Thus, the hybrid enzyme can be used as an anti-inflammatory target. A cell-based assay was used to test the COX-2 inhibitor targeting the hybrid enzyme for reducing inflammatory PGE2 production. Figure 8 shows that the cells overexpressing the COX-2-10aa-mPGES-1 converted the \(^{14}\)C-AA into \(^{14}\)C-PGE2 (Fig. 8A), and were completely inhibited by the presence of the COX-2 inhibitor (Fig. 8B). This finding clearly indicates that the hybrid enzyme cells could be used for further NSAID screening which targets the suppression of PGE2 production mediated by the inducible COX-2 and/or mPGES-1.

Discussion

The end-products, prostanoids metabolized from AA through the COX-pathway, play important roles in balancing
hemostasis, vascular functions, inflammation and cancer development. The unbalanced biosynthesis of prostanoids is involved in many critical diseases, such as stroke, myocardial infarction, inflammation and cancers. The controlling and re-directing of the prostanoids' biosyntheses in cells through the COX-pathway is a potential approach in correcting this imbalance. Our recent discovery of a specially engineered hybrid enzyme that links COX to PGIS could effectively control and re-direct the AA to be metabolized into PGI2 for vascular protection (Ruan et al., 2006, 2008a, 2008b). This finding has led us to hypothesize that the COX-pathway in cells could be re-directed and controlled by overexpressing designed hybrid enzymes linking COX to different downstream synthases. To validate this new concept, it is critical to prove that aside from PGIS, other downstream synthases,

![Figure 5](image1.png)

**Fig. 5.** Determination of the tri-catalytic activities of the recombinant proteins directly converting AA to PGE2 using enzyme immunoassay (EIA). Following the enzyme reactions (described in Fig. 3) for the reaction mixtures prepared from the cells transfected with the plasmid of the COX-2-10aa-mPGES-1 (A), the mixture of the plasmids of the COX-2 and mPGES-1 (B), or the pcDNA3.1 vector alone (C), the samples were diluted 100 times with PBS containing 0.1% bovine serum albumin (BSA), and then used for quantitative determination of PGE2 using an EIA kit following the instructions of the manufacturer (Cayman, Ann Arbor, MI). A PGE2 standard is shown in column (D).

![Figure 6](image2.png)

**Fig. 6.** Determination of the triple catalytic activities of the recombinant proteins directly converting DGLA to PGE1 using an HPLC method for HEK293 cells. The [14C]-DGLA (10 μM) was added to the cells expressing COX-2-10aa-mPGES-1 (A). The [14C]-PGE1 standard produced is shown in (B). The methods used for assay and HPLC analysis were described in Fig. 4.

![Figure 7](image3.png)

**Fig. 7.** (A) Time-course of the conversion of AA to PGE2 by the recombinant proteins. The conversion of AA to PGI2 by the HEK293 cells expressing COX-2-10aa-mPGES-1 (open circles) or those co-expressing COX-2 and mPGES-1 (closed squares) was performed with increasing times using the HPLC method as described in Fig. 4. The amount of [14C]-PGE2 produced at increasing reaction times were calculated and plotted as percentages of the added substrate, [14C]-AA (10 μM). (B) Enzyme kinetic properties of the expressed recombinant proteins. Increasing concentrations of [14C]-AA were added to the HEK293 cells expressing COX-2-10aa-mPGES-1 (open circles) or co-expressing COX-2 and mPGES-1 (closed squares) for 60 s. The reactions were terminated and then analyzed by HPLC. The conditions, including protein concentration and buffers used, were identical to that of Fig. 4.
such as mPGES-1, cytosolic PGES (cPGES), PGD2 synthase (PGDS), PGF2 synthase (PGFS) and TXA2 synthase (TXAS), could also be linked to COX to form active triple catalytic enzymes. This would be particularly interesting for mPGES-1, which is quite different than the microsomal P450 enzyme, PGIS. Engineering the active COX-2-10aa-mPGES-1 described in results has provided evidence to support that COX is an enzyme which can be linked to its downstream substrates with different structures and topologies, and re-direct the prostanoid biosyntheses in general. From a protein structural point of view, the current studies have also evidenced that the C-terminus of COX-2 and the N-terminus of mPGES-1 are flexible and could be modified without changing the enzymes' catalytic functions. Furthermore, because mPGES-1 belongs to the Membrane Associated Proteins in Eicosanoid and Glutathione (MAPEG) enzyme family (Jakobsson et al., 1999), which includes the two other AA-metabolizing enzymes, 5-lipoxygenase activating protein (FLAP) and leukotriene C4 synthase (LTC4S), it may be suggested that these two enzymes could also be modified at N-terminal positions.

COX-2 and mPGES-1 are inducible enzymes, expressed in response to the critical physiological processes, including cell proliferation and differentiation, and tissue regeneration and repair. On the other hand, a process that might be involved in the pathological processes of inflammation and cancers is the overexpression of the inducible COX-2 associated with mPGES-1, which produces PGE2 from AA. Thus, understanding the coupling mechanism between the COX-2 and mPGES-1 in the biosynthesis of PGE2 is a key step toward developing a therapeutic intervention to control and re-direct the coupling between COX-2 and mPGES-1. Previous studies have described that COX-2 couples to mPGES-1 but not cPGES through the co-expression of the recombinant COX-2 and mPGES-1 in HEK293 cells (Murakami et al., 2002). However, little information is available to further demonstrate this favored coupling at the molecular level. The results described in Fig. 7A show that the initial velocity of the coupling reaction of the cells expressing COX-2-10aa-mPGES-1 and co-expressing COX-2 and mPGES-1 are similar. Here, enough evidence is provided to suggest the movement of PGH2 from COX-2 to mPGES-1, which have similar distances in the linked and unlinked COX-2 and mPGES-1. It is also suggested that the proximity between the individual COX-2 and mPGES-1 in the ER membrane is similar to that of the COX-2-10aa-mPGES-1, in which the two enzymes are separated by approximately 10 Å (through the 10aa linker). It becomes obvious that PGH2, produced by the COX-2 traveling from the ER lumen to the cPGES in the cytoplasm, has to travel a much longer distance than 10 Å. The observation of the shorter distance between COX-2 and mPGES-1 than that of cPGES could be one of the mechanisms useful in explaining the selectivity of the coupling between COX-2 and mPGES-1 in the cells.

On the other hand, it has been observed that PGE2 is a dominant product metabolized from AA when COX-2 and mPGES-1 are induced, other COX-downstream enzymes are expressed in inflammation and cancers. As shown in Fig. 7B, the low $K_m$ value could be one of the mechanisms for the favored coupling between the COX-2 and mPGES-1 in the pathological processes since most other COX-downstream enzymes have higher $K_m$ values (Thorén et al., 2003) than this.

COX-2 and mPGES-1 are important targets for developing anti-inflammatory drugs. The cells expressing the engineered COX-2-10aa-mPGES-1, which specifically convert AA into PGE2, can be used as an effective model for initial drug screening in search of the effects of the chemical compounds, genetic molecules and regulators on PGE2 production. In addition, the COX-2-10aa-mPGES-1 gene could be used to produce transgenic mice with an overproduction of PGE2 for further uncovering PGE2 functions from the early embryo development to the later inflammation responses and cancers in vivo.

Acknowledgements

We thank Dr Richard J. Kulmacz for providing the original wild-type cDNA of human COX-2.

Funding

This work was supported by the National Institutes of Health (HL56712, HL79389 to K.H.R.).
References


Received May 8, 2009; revised September 8, 2009; accepted September 9, 2009

Edited by Anthony Wilkinson