Published in final edited form as:

Arch Biochem Biophys. 2008 December 1; 480(1): 41–50. doi:10.1016/j.abb.2008.09.010.

Large-scale expression, purification, and characterization of an engineered prostacyclin-synthesizing enzyme with therapeutic potential

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Abstract

Recently, we reported that a novel hybrid enzyme (TriCat Enzyme), engineered by linking human cyclo-oxygenase-2 (COX-2) with prostacyclin (PGI₂) synthase (PGIS) together through a transmembrane domain, was able to directly integrate the triple catalytic (TripCat) functions of COX-2 and PGIS and effectively convert arachidonic acid (AA) into the vascular protector, PGI₂ (Ruan, KH., et al., 2006, *Biochemistry*, (47):14003–11). In order to confirm the important biological activity and evaluate its therapeutic potential, it is critical to characterize the properties of the enzyme using the purified protein. The TriCat Enzyme cDNA was subcloned into a baculovirus vector and its protein was expressed in Sf-9 cells in large-scale with a high yield (~4% of the total membrane protein), as confirmed by Western blot and protein staining. The Sf-9 cells' membrane fraction, rich in TriCat Enzyme, exhibited strong TriCat functions ($K_m = 3 \mu\text{M}$ and $K_{cat} = 100$ molecules/min) for the TriCat enzyme and was 3-folds faster in converting AA to PGI₂ than the combination of the individual COX-2 and PGIS. Another superiority of the TriCat Enzyme is its dual effect on platelet aggregation: it completely inhibited platelet aggregation at the low concentration of 2 $\mu\text{g/ml}$ and then displayed the ability to reverse the initially aggregated platelets to their non-aggregated state. Furthermore, multiple substrate-binding sites were confirmed in the single protein by high-resolution NMR spectroscopy, using partially purified TriCat enzyme. These studies have clearly demonstrated that the isolated TriCat Enzyme protein functions in the selective biosynthesis of the vascular protector, PGI₂, and revealed its potential for anti-thrombosis therapeutics.

Keywords

Prostacyclin; Prostaglandin 12; Cyclooxygenase; Protein engineering; Prostacyclin synthase; Vascular protector; Anti-thrombosis

The biosynthesis of prostanoids (prostaglandins and thromboxane) occurs via the COX¹ pathway from arachidonic acid (AA) in three/triple catalytic steps [1–5] by COX and its downstream synthases: AA, released from the membrane phosphoglycerides, is converted to the prostaglandin G₂ (PGG₂, catalytic step 1), and then to the prostaglandin endoperoxide (prostaglandin H₂ (PGH₂)) (catalytic step 2) by COX isoform-1 (COX-1) or COX-2. PGH₂ is

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¹Abbreviations used: COX, cyclooxygenase; PGI₂, prostacyclin or prostaglandin I₂; PGIS, PGI₂ synthase; TXA₂, thromboxane A₂; TXAS, TXA₂ synthase; PCR, polymerase chain reaction; AA, arachidonic acid; HPLC, high-performance liquid chromatography; PRP, platelet rich plasma; BSA, bovine serum albumin; U46619, 9,11-Dideoxy-9 α ,11 α -methanoepoxyprostaglandin F₂ α .

further isomerized to the biologically active end-products, prostaglandin D₂ (PGD₂), E₂ (PGE₂), F₂ (PGF₂), and I₂ [PGI₂ (prostacyclin)] or thromboxane A₂ (TXA₂) by individual synthases (catalytic step 3) in tissue-specific processes. TXA₂, produced from PGH₂ by TXA₂ synthase (TXAS), has been implicated as a proaggregatory and vaso-constricting mediator which is one of the major factors causing stroke and heart attacks [6–7]. PGI₂ is the primary AA metabolite in vascular walls and has opposing biological properties to that of TXA₂ and therefore represents the most potent endogenous vascular protector by acting as an inhibitor of platelet aggregation [8] and a strong vasodilator on vascular beds [9–12]. A selective increase in the production of PGI₂ with a decrease in TXA₂ production is the ideal outcome for a model that prevents and protects against vascular diseases, including thrombosis, hypertension, strokes and heart attacks. From the century-old aspirin to the more recently developed COX-2 inhibitors, the drugs have not yet achieved this goal. Finding a way to specifically increase the production of the vascular protector, PGI₂, will be one of the most attractive therapeutic developments in pharmaceutical intervention. Recently, we have successfully engineered an innovative enzyme by linking COX-2 and PGIS together to form a single protein using a molecular cloning approach [13]. The engineered enzyme narrowly expressed in HEK293 and COS-7 cells could actively convert AA into PGI₂ through its triple catalytic activities. As a result, this engineered enzyme has been reported as a “Super Enzyme” with great potential for fighting heart disease by the American Chemical Society [14]. In this paper, the active Triple Catalytic (TriCat) Enzyme was first successfully produced in large-scale using a baculovirus (BV) system, and its benefits for specifically up-regulating PGI₂ biosynthesis and its potent anti-thrombosis properties, which are superior to that of the individual COX-2 and PGIS, were confirmed and further characterized. The studies have revealed a great potential for developing the innovative protein into a new therapeutic intervention for vascular disease requiring increased levels of PGI₂.

Experimental procedures

Materials

COS-7 and HEK293 cell lines were purchased from ATCC (Manassas, VA). Medium for culturing the cell lines was purchased from Invitrogen. [¹⁴C]-Arachidonic Acid (AA) was purchased from Amersham Biosciences. The non-labeled AA for the platelet aggregation studies was purchased from Chrono-Log Corp., (Havertown, PA.) and Bio Data Corp., (Horsham, PA). The aspirin used in this experiment was purchased from Sigma.

Engineering of the TriCat Enzyme cDNA containing the sequences of COX-2 and PGIS

The cDNA of the newly engineered TriCat Enzyme (COX-2-10aa-PGIS) was previously described (Ruan et al., 2006). Briefly, COX-2 was linked to a 10 amino acid TM linker connected to PGIS. COX-2 was generated by a PCR approach and then subcloned into a pcDNA3.1 vector with a CMV promoter. The resultant pcDNA3.1-COX-2-10aa-PGIS was used for expressing the TriCat Enzyme in HEK293 and COS-7 cells.

Subcloning of the COX-2-10aa-PGIS cDNA into the baculovirus (BV) vector

First, a BamH I insert was added to the front of the COX-2-10aa-PGIS cDNA's start codon, and then a 6His-tag sequence was inserted between N19 and P20 of the COX-2 sequence in the pcDNA3.1 vector using a PCR approach. The cDNA of the 6His-COX-2-10aa-PGIS (6His-TriCat Enzyme) was cut from the pcDNA3.1 vector using EcoR I and BamH I restriction enzymes and then inserted into the BV-suitable vector, pVL1392, to obtain the final pVL1392-6His-TriCat Enzyme construct.

Packing the BV for expression of the 6His-TriCat Enzyme in Sf-9 cells and large scale-expression

The packing of the BV for high-yield expression of the TriCat Enzyme in the Sf-9 insect cells was performed in two steps. 1.) The Sf-9 cells were co-transfected with 2.0 µg of the pVL1392-6His-TriCat Enzyme plasmid and 0.5 µg of Orbigen Sapphire DNA using a calcium phosphate precipitation method; 2.) Six days after the co-transfection, the virus-containing supernatant was harvested and plaqued at dilutions of 10^{-5} , 10^{-6} and 10^{-7} . One week later, ten plaques (A-J) were selected and each viral isolate was put into 1.0 ml of serum free Grace's media overnight. The following day, 0.5 mL of each viral plaque was added to an individual T25 flask of Sf-9 insect cells. After infection, the cells were harvested at different times to view the expression levels of the 6His-TriCat Enzyme. The specific plaque (plaque H) of the transfected Sf-9 cells with high-yield expression of the TriCat Enzyme was selected for further middle-scale (500 mL) and large-scale (5 L,) expression, both of which were performed in the Baculovirus/Monoclonal Antibody Facility, Baylor College of Medicine (Houston, TX).

Expression and Co-expression of the individual COX-2 and PGIS in COS-7 or HEK293 Cells

The recombinant synthases were expressed in COS-7 or HEK293 cells as described [15–16]. Briefly, the 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, then grown at 37 °C in a humidified 5% CO₂ incubator. The cells were then transfected with a purified cDNA of the recombinant protein by the Lipofectamine 2000 method [17] following the manufacturer's instructions (Invitrogen). Approximately 48 hrs after transfection, the cells were harvested for further enzyme assay and a Western blot was performed. For the co-transfection, a 3:2 ratio of the cDNAs of COX-2 and PGIS were used.

Preparation of microsomal and membrane fractions of the Sf-9 cells

The general procedure for microsomal preparation was previously described [18]. The transfected or untransfected Sf-9 cells were harvested into ice-cold PBS buffer, pH 7.4, and collected by centrifugation. After the cells were washed three times, the pellet was resuspended in a small volume of the same buffer, sonicated briefly, and centrifuged at 10 000g for 10 min. The supernatant was collected and centrifuged at 200 000g for 40 min to pellet the microsomal and membrane fractions [18].

Electrophoresis and Western blot

Membrane proteins were separated by 7% (w/v) SDS-PAGE under denaturing conditions and then transferred to a nitrocellulose membrane. Bands recognized by specific primary antibodies against human PGIS or COX-2 were visualized with horseradish peroxidase-conjugated secondary antibody and chromogenic peroxidase substrates [13]. The intensities of the bands in the immuno-blot were used to normalize the enzyme activities as well.

Enzyme Activity Determination for COX-2 and PGIS using the HPLC-scintillation analysis method [13]

To determine the activity of the enzyme that converted AA into PGI₂, the membrane protein was incubated with different concentrations of [¹⁴C]-AA (0.3–60 µM) in a total reaction volume of 30–100 µL. After a 0.5–5 min incubation, the reaction was terminated by adding 200 µL of the solvent containing 0.1% acetic acid and 30% acetonitrile (solvent A). After centrifugation (12 000 rpm for 5 min), the supernatant was injected into a reverse phase C18 column (Varian Microsorb-MV 100-5, 4.5 × 250 mm) using the solvent A with a gradient from 35 to 100% of acetonitrile for 45 min at a flow-rate of 1.0 mL/min. The [¹⁴C]-labeled AA metabolites, including [¹⁴C]-6-keto-PGF_{1α} (degraded PGI₂) were monitored directly by a flow scintillation analyzer (Packard 150TR). The peak and the relative amount of [¹⁴C]-6-keto-

PGF_{1α} converted from [¹⁴C]-AA was confirmed and calibrated by 6-keto-PGF_{1α} standards using Enzyme Immunoassays as described [13].

Anti-Platelet Aggregation Assays

A sample of fresh blood was collected using a collection tube with 3.2% sodium citrate for anticoagulation. The platelet-rich plasma was separated from the blood by low speed centrifugation. A total of 0.5 mL of the platelet-rich plasma was incubated with either saline, membrane protein or cells (HEK293 or Sf-9) at 37 °C for 3 min. Sodium arachidonate (final concentration 5 mg/mL) was added to the platelet sample and the platelet aggregation was monitored by the anticoagulation analyzer (model 490, Chronolog Aggregometer with AggregoLink software; Havertown, Pennsylvania). Platelet aggregation was determined by conventional light transmittance aggregometry, in which aggregation was expressed as the maximal percent change in light transmittance from baseline, using the platelet-poor plasma as a reference.

Purification of the TriCat Enzyme

A 500 mL suspension culture of the Sf-9 cells expressing the TriCat enzyme was washed twice with ice-cold PBS and collected by centrifugation. The membrane fraction was separated by freeze-thaw of the cells in PBS containing 1 mM PMSF. The enzyme was further purified by solubilization of the membrane fraction using 1% octyl glucoside overnight and ultracentrifugation at 100,000g at 4 °C. The supernatant was purified by a Superdex-75 gel filtration column (1 cm × 45 cm) with a flow rate of 0.2 mL/min using PBS with 0.1% octyl glucoside. The fractions containing the active enzyme protein were identified by activity assays, SDS–PAGE analysis, and Western blot analysis.

NMR experiments

The tr-NOE NMR experiment, which determined the interaction of the partially purified TriCat Enzyme (final concentration of 5.2 mM) with the ligands, U46619 (PGH₂ mimic) and ibuprofen (AA analogue), was performed according to the procedures described previously [19]. Briefly, the 2D DQF-COSY, TOCSY, and NOESY spectra were taken for U46619 and ibuprofen (4 mM) in the absence and presence of the partially purified TriCat Enzyme (1 mg/0.5 ml, in 20 mM sodium phosphate buffer, pH 7.5, containing 9% ethanol-*d*₆ and 9% D₂O) and then recorded. All NMR experiments were carried out on a Bruker Avance 600 MHz NMR spectrometer with a 5 mm triple-resonance probe at 298 K. The water peak was suppressed by the excitation sculpting method [20]. All 1D spectra contain 16K data points. NOESY and TOCSY spectra contain 2048 × 512 data points, and the DQF-COSY spectra contain 4096 × 512 data points. The NOESY spectra were recorded with a mixing time of 150 and 300 ms. The TOCSY spectra were carried out with an MLEV-17 spin-lock pulse sequence with a total mixing time of 70 ms. Quadrature detection was achieved in F1 by the states-TPPI method. The NMR data were processed using the Felix 2000 program (Accelrys, San Diego, CA).

Results

Designing and Subcloning of the TriCat Enzyme's cDNA into the baculovirus (BV) vector for high yield- large scale expression

Our previous characterization of the engineered TriCat Enzyme, which integrated triple catalytic functions into one protein molecule (Fig. 1), was limited by the low expression system in HEK293 and COS-7 cells [13]. To further reveal the TriCat Enzyme's properties and explore its potential for therapeutic uses, it is crucial to establish a method for the large-scale expression of the TriCat Enzyme, and to identify the expressed enzyme in an active form. The BV expression system has been widely used to prepare high-yield expression systems for

recombinant proteins. It has also been reported that human COX-2 with a 6His-tag inserted on the N-terminal domain (after the signal peptide) can be actively expressed in Sf-9 cells [21]. Following the report and procedures provided from the manufacturer of the BV vector [22–23], the insert of the cDNA of the active TriCat Enzyme was prepared from the pcDNA3.1-COX-2-10aa-PGIS plasmid [13] and then a 6His-tag was added onto the N-terminal domain of the TriCat Enzyme after the COX-2 signal peptide for future purifications using a PCR technique.

Large-scale expression of the 6His-TriCat Enzyme using the BV expression system

The constructed pVL1392-6His-TriCat Enzyme was used to express 6His-TriCat Enzyme in Sf-9 cells. In the initial expression studies, five of the ten plaques from the BV-transfected Sf-9 cells (5 ml each) were able to express the 6His-TriCat Enzyme with a high yield (Fig. 2A). It was interesting that no degraded fragments of the highly overexpressed 6His-TriCat Enzyme were observed in any of the positive Sf-9 cell plaques (Fig. 2A), which indicated that the majority of the expressed 6His-TriCat Enzyme has a correct protein folding and membrane insert in the cells. The enzyme assay has indicated that all of the five positive plaques of the Sf-9 cells show strong activity as well (Fig. 2B). Amongst the positive plaques, the most active 'H' plaque of the Sf-9 cells (Fig. 2A and B) was selected for a time course investigation to further optimize the expression yield. The Western blot results from this experiment showed that the expression yield of the 6His-TriCat Enzyme reached a maximum at 64-hours, and had no significant change up to 72-hours (Fig. 2C). Thus, the clone set up for 72-hours expression was used for modulate-scale (500 mL) and large-scale (5 L) expression of the 6His-TriCat Enzyme. The yield of the 6His-TriCat Enzyme expression was approximately 4% of the total protein, as evidenced by Coomassie Blue staining (Fig. 2D) and Western blot (Fig. 2E).

Triple catalytic activities of the 6His-TriCat Enzyme and its comparison with that of the wild type TriCat Enzyme expressed in mammalian cells

Some proteins, particularly the membrane proteins with a high-yield expression in Sf-9 cells, could become misfolded and result in the unwanted production of inactive protein. Therefore, it is very important to test whether the membrane-bound 6His-TriCat Enzyme prepared from the large-scale expression could uphold its correct folding and the triple catalytic activities for directly converting AA to PGI₂, as this data is compatible with that of the wild type TriCat Enzyme (without a 6His tag) expressed in the mammalian cells, HEK293 and COS-7 cells as described [13]. The expressed enzyme was activated by the addition of [¹⁴C]-AA as an initial substrate and the metabolized products were monitored by an HPLC-scintillation analyzer. The single product, [¹⁴C]-6-keto-PGF_{1α} (degraded from [¹⁴C]-PGI₂) was observed in the analysis profile for the Sf-9 cells expressing the 6His-TriCat Enzyme (Fig. 3, top panel). In contrast, a single un-metabolized [¹⁴C]-AA peak was observed in that of the assay using control Sf-9 cells (Fig. 3, lower panel). These results indicated that no endogenous COX activity was present in the Sf-9 cells alone, and the [¹⁴C]-AA was almost completely converted by the 6His-TriCat Enzyme expressed in the Sf-9 cells into [¹⁴C]-PGI₂. This requires the following three-step catalytic reactions by the single protein: 1) [¹⁴C]-AA catalyzed into [¹⁴C]-PGG₂ by the COX's cyclooxygenase activity; 2) [¹⁴C]-PGG₂ catalyzed into [¹⁴C]-PGH₂ by the COX's peroxidase activity, and 3) [¹⁴C]-PGH₂ isomerized to [¹⁴C]-PGI₂ by the PGIS activity (Fig. 1). The specific enzyme activity of the 6His-TriCat Enzyme expressed in the Sf-9 cells was further compared with that of the wild type TriCat enzyme expressed in the mammalian cell line, HEK293. Given the same amount of crude membrane protein, the amount of PGIS produced (shown as [¹⁴C]-6-keto-PGF_{1α}) from 6His-TriCat Enzyme was approximately 100-fold more than that of the wild type TriCat Enzyme, which also demonstrated approximately 100-fold less in expression yield (Fig. 4). Based on the data the expression yield of TriCat enzyme in HEK293 and COS-7 cells was approximately 0.04% of the total protein. This has clearly shown that the 6His-TriCat Enzyme expressed by the BV-system has a specific enzyme activity similar to that of the wild

type TriCat Enzyme expressed in the HEK293 cells. Thus, the established BV-expression system is suitable for large-scale production of the active TriCat Enzyme, and the 6His-TriCat Enzyme could be used for the further characterization of the biological activity of the TriCat Enzyme in general.

The TriCat Enzyme's superiority in the selective production of PGI₂ from AA compared to the mixture of the individual COX-2 and PGIS enzymes

Once the endogenous and exogenous AA is converted to PGH₂ by cyclooxygenase, PGH₂ is presented to the downstream synthases, such as PGIS, to be isomerized into PGI₂. However, during its travel from COX to the downstream enzymes, the unstable PGH₂ can be degraded into non-enzymatic products, such as PGF_{2α} and HHT (Fig. 5A). One of the predicted superiorities of the TriCat Enzyme is that it should be able to reduce PGH₂ degradation by shortening the time and traveling distance for PGH₂ movement from the COX catalytic site to the PGIS catalytic site. An activity assay has been used to test this hypothesis, in which the membrane-bound 6His-TriCat Enzyme (prepared from the Sf-9 cells) was able to convert the majority of [¹⁴C]-AA into [¹⁴C]-6-keto-PGF_{1α} in five minutes and very little degraded PGH₂ production was observed (Fig. 5B). In contrast, significant [¹⁴C]-HHT and other degraded products from the [¹⁴C]-AA were observed when using the co-expressed COX-2 and PGIS (Fig. 5C). The inhibition of the COX-2-10aa-PGIS activity was previously determined using specific COX-2 and PGIS inhibitors [13]. These results provided the evidence that shows the TriCat Enzyme is indeed able to reduce PGH₂-degraded products and increase the selectivity for producing PGI₂ more efficiently than the individual enzyme mixture of COX-2 and PGIS. It also implied that the PGH₂ has less of a chance to be used by other downstream synthases, such as TXAS and PGES.

Kinetic studies demonstrating the superiorities of the TriCat Enzyme in converting AA to PGI₂

1) A faster initial velocity: The membrane-bound 6His-TriCat Enzyme displayed a much faster initial reaction kinetic compared to the mixture of the individual COX-2 and PGIS anchored in the ER membrane. To reach 50% of the maximum activity, the 6His-TriCat Enzyme only needed 20 seconds (Fig. 6A), but the co-expressed individual COX-2 and PGIS required 60 seconds (Fig. 6B). This indicates that the TriCat Enzyme is 3-folds faster in converting AA into PGI₂ than that of the mixture of the individual enzymes. 2) Km values: In our assay system, we have found that the Km values for the membrane-bound 6His-TriCat Enzyme's direct conversion of AA into PGI₂ is approximately 3.0 μM (Fig. 6C), which is similar to wild-type TriCat Enzyme expressed in HEK293 cells (3.9 μM, [13]). This value is significantly less than the reported PGIS Km values of 9 [24], 13 [25], and 30 [26] μM, and similar to the reported COX-2 Km values (Table 1), indicating that the TriCat Enzyme has an advantage over the mixture of the COX-2 and PGIS for PGI₂ biosynthesis. 3) K_{cat} (V_{max}) value: The K_{cat} (V_{max}) value for the biosynthesis of PGI₂ (from AA) is reduced by the two individual enzymes' coupling processes, but it is possibly increased by the single protein - TriCat Enzyme. The K_{cat} for the co-expressed individual COX-2 and PGIS (COX-2/PGIS) is only 20 mole of PGI₂/min/mole enzyme (data not shown). However, for the 6His-TriCat Enzyme, the value increases to 100 mole of PGI₂/min/mole enzyme (Fig. 6C). Therefore, this data clearly indicates that the TriCat Enzyme could produce PGI₂ much faster (5-folds) than that of the individual COX-2 and PGIS.

Superiority of the TriCat Enzyme on anti-platelet aggregation through the dual effects (increasing PGI₂ production while decreasing TXA₂ synthesis)

The fast and selective conversion of AA to PGI₂ by the fusion enzyme as described above has suggested that it could also decrease the production of the thrombosis-mediator, TXA₂,

produced from the same precursor, AA, from circulating platelets which contain COX-1 and a large amount of TXAS. This means that the TriCat Enzyme shall be a potent anti-platelet aggregation reagent through its dual effects on anti-platelet aggregation in anti-thrombosis events (one effect is an increased PGI₂ production and the other is a reduced TXA₂ production). To test this hypothesized superiority of the TriCat Enzyme, the AA-induced platelet aggregation experiment was performed in the presence of membrane-bound 6His-TriCat Enzyme (Fig. 7I, A), a mixture of the membrane-bound individual COX-2 and PGIS co-expressed in HEK293 cells (Fig. 7I, B), or Sf-9 cell membrane protein (Fig. 7I, C). After the addition of AA to the platelet-rich plasma (PRP), the endogenous COX-1 and TXAS (from the platelets) converted AA into TXA₂, which quickly induced platelet aggregation (Fig. 7I, D). A similar result was also seen by the addition of the Sf-9 cell membrane protein as the control (Fig. 7I, C). However, the induced platelet aggregation was completely blocked by the addition of a small amount of TriCat Enzyme (Fig. 7I, A), whereas only minimal effects were seen on the blockage by the addition of an equal amount of COX-2 and PGIS proteins (Fig. 7I, B). After monitoring the 6His-TriCat Enzyme-mediated inhibition of the platelet aggregation for up to 20 minutes, non-reversible platelet aggregation was observed (data not shown). This revealed that the inhibition of the platelet aggregation by the TriCat Enzyme has a long-lasting effect. A dose-dependent inhibition of the platelet aggregation by the 6His-TriCat Enzyme was also observed, in which the IC₅₀ was approximately 3 µg/mL (Fig. 7II).

Superiority of the TriCat Enzyme on initial anti-platelet aggregation – reversing the AA-induced platelet aggregation

To further characterize the anti-thrombosis properties, a reversal platelet aggregation assay was performed. The platelet aggregation was induced by AA first (Fig. 8I). Following the platelet aggregation the 6His-TriCat Enzyme was added to the PRP sample (Fig. 8I, A). Surprisingly, the TriCat Enzyme was able to reverse the state of the aggregated platelets to an un-aggregated state within a few minutes after the addition of the enzyme to the aggregated platelets (Fig. 8I, A). The co-expressed COX-2 and PGIS in HEK293 cells showed very little activity in reversing the platelet aggregation (Fig. 8I, B). Even the most powerful blood thinner, aspirin, failed to reverse the platelet aggregation (Fig. 8I, C). The Sf-9 cell protein (control) also had no effect on reversing aggregation (Fig. 8I, D). These results strongly support the hypothesis that the TriCat Enzyme could be useful for the initial thrombosis mediated by platelet aggregation.

The Dual effects of the TriCat Enzyme on anti-platelet aggregation

The molecular mechanisms of how the TriCat Enzyme strongly inhibited the AA-induced platelet aggregation and reversed the initially aggregated platelets to a non-aggregated state were further investigated. A trace amount of [¹⁴C]-AA was mixed with unlabeled AA to induce the platelet aggregation in the presence and absence of the membrane-bound TriCat Enzyme or the mixture of membrane-bound COX-2 and PGIS as described in Fig. 7II. The metabolized [¹⁴C]-AA was profiled by HPLC-scintillation analysis, which clearly showed that in the absence of the 6His-TriCat Enzyme, a large amount of [¹⁴C]-AA was converted to [¹⁴C]-TXA₂ (shown as [¹⁴C]-TXB₂ peak at 10 min) and induced platelet aggregation by the endogenous COX-1 and TXAS from the platelets (Fig. 8II, A). However, in the presence of the 6His-COX-2-10aa-PGIS, the [¹⁴C]-TXB₂ peak disappeared and only [¹⁴C]-6-keto-PGF_{1α} was observed in the assay (Fig. 8II, B). In contrast, in the presence of the mixture of COX-2 and PGIS, the [¹⁴C]-TXA₂ peak was still present and the [¹⁴C]-6-keto-PGF_{1α} peak was much smaller than that of the 6His-TriCat Enzyme (Fig. 8II, C). These results provided direct evidence demonstrating how the 6His-TriCat Enzyme was not only able to directly convert the AA to PGI₂, but could also maintain its capacity to compete with the endogenous COX-1/TXAS to reduce the TXA₂ production. The dual effects are the molecular basis for the

TriCat Enzyme's superiorities in anti-platelet aggregation and anti-thrombosis compared to that of the individual COX-2 and PGIS.

Characterization of the TriCat functions of the COX-2-10aa-PGIS using the NMR spectroscopic technique

To identify the multiple substrate binding sites in the COX-2-10aa-PGIS, the 6-His-TriCat enzyme was partially purified by detergent extraction, ultracentrifugation and gel filtration. Because of the BV expression system's high yield (Fig. 2) and the unique molecular weight (135 kDa), the 6-His-TriCat enzyme was easily isolated from the membrane fraction of the Sf-9 cells using the following three steps: 1) octyl glucoside extraction; 2) ultracentrifugation and 3) gel filtration. The 6-His-TriCat enzyme was purified to approximately 70% purity (Fig. 9A) and retained more than 90% of its TriCat activities (Fig. 9B). The transfer-NOE (trNOE) NMR spectroscopy, which required only partially purified protein, was used to determine the AA and PGH₂ binding sites in the 6-His-COX-2-10aa-PGIS TriCat enzyme. Binding of both ligands, Ibuprofen (AA mimic) and U46619 (PGH₂ mimic) was evidenced by the following characteristics: 1) The NOEs of both, ibuprofen and U46619, changed from the "negative phase," represented by the free form small ligands (green color, Fig. 10A, C) to the "positive phase," represented by the ligands bound to the large protein molecule, COX-2-10aa-PGIS (grey color, Fig. 10B and D); and 2) The molecules, ibuprofen and U46619 underwent conformational changes because several intramolecular NOEs of the ligands were clearly observed (Fig. 10B and D (boxed NOEs)). The binding of U46619 to purified PGIS in solution determined by the NMR technique was used as a positive control [27]. This experiment has provided detailed information and direct evidence supporting the conclusion that the engineered COX-2-10aa-PGIS contains AA and PGH₂ binding sites.

Discussion

The successful high-yield expression of the active 6His-COX-2-10aa-PGIS, using the BV system described in the studies (Fig. 2 and Fig. 3), has proven that the engineered enzyme could be expressed in large-scale, which is essential for the detailed characterization of the enzyme properties and biological activities. The studies have also proven that the expressed 6His-COX-2-10aa-PGIS has triple catalytic activities, which are similar to that of the enzyme expressed in the mammalian cells, such as HEK293 and COS-7 cells as reported [13]. With this information on hand, further characterization of the engineered enzyme becomes possible when using structural biology, spectroscopy and animal trials, all of which require large amounts of the active enzyme.

A key element for the successful engineering of this active TriCat Enzyme was the ability to mimic the native membrane environment of the COX and PGIS, which are both anchored to the ER membrane. To evaluate its biological functions implied in the native cells' environment, it is critical to demonstrate the triple catalytic activities for the 6His-TriCat Enzyme in the membrane-bound environment. These studies used the membrane-bound 6His-TriCat Enzyme for the characterization of the enzyme properties and superiorities in anti-platelet aggregation and compared them to that of the membrane-bound COX-2 and PGIS. The results gave direct evidence showing how the triple catalytic reactions of the engineered enzyme worked in conditions that mimicked that of the cells and tissues. It also led to the prediction that the TriCat Enzyme could remain active once successfully delivered to or spread in the animal tissues. Therefore, the TriCat Enzyme should be able to convert the over-released free AA into PGI₂ if the TriCat Enzyme could be expressed in human cells and delivered into the circulation system. Of course, to make any conclusions, intensive investigations for the expression of the TriCat Enzyme in human cells and delivery methods are needed.

For decades now, one of the major challenges for medical and pharmaceutical researchers has been to find a specific way to up-regulate the production of vascular protectors, such as PGI₂, and/or specifically down-regulate the production of the thrombosis mediators and vasoconstrictors, such as TXA₂. Unfortunately, little information is available for such drugs or therapeutic approaches. This dual effect of up-regulating PGI₂ biosynthesis while decreasing TXA₂ production, exhibited by the engineered enzyme's activities (Fig. 7 and Fig. 8) has turned it into a very interesting molecule that could represent a new generation of therapeutic, anti-thrombosis intervention. In addition, an excess amount of free AA in the blood circulation is a considerable risk for thrombosis because the circulating platelets rich in COX-1 and TXAS could instantly convert AA to TXA₂ and trigger stroke, thrombosis and heart disease. The TriCat enzyme, with its dual functions, could be a powerful enzyme to help reduce the free AA in circulation and maintain the remaining AA at a low concentration for the prevention of vascular disease. It was also very interesting to find that the TriCat enzyme was able to reverse the platelet aggregation, which is something that could not be accomplished with aspirin, one of the most popular drugs used in the prevention and treatment of thrombosis (Fig. 8). These results strongly imply that the hybrid enzyme can not only inhibit further platelet aggregation, but it can also dissolve the aggregated platelets in the initial stages of thrombosis, which shall be further tested in vivo.

Fig. 9B has shown that the partially purified 6-His-TriCat enzyme, in the presence of 1% octyl glucoside, retained more than 90% activity. This finding suggests that: 1) The protein folding of the TriCat Enzyme in non-ionic detergent is similar to that of its membrane-bound form; and 2) Further determination of the protein and ligand interaction can be done in the presence of the non-ionic detergent. This has provided a basis for further characterization of the AA and PGH₂ binding sites in the single molecule (TriCat enzyme) using high-resolution NMR spectroscopy. Recently, we have developed methods to characterize the substrate mimics' interaction with purified eicosanoid enzymes in solution using the transferred NOE technique [19,27]. These same methods were adopted for the TriCat Enzyme studies. Fig. 10 has clearly indicated that both, the AA and PGH₂ mimics bound to the partially purified TriCat Enzyme, as evidenced by the conformational changes of the ligands.

COX-2-induced inflammation and pain occurs primarily through its coupling, COX-2's coupling, to the inducible microsomal PGE₂ synthase-1 (mPGES-1), which overproduces PGE₂. On the contrary, the TriCat Enzyme may not only increase PGI₂ synthesis from AA, but by doing so would also reduce the amount of AA available to mPGES-1 and prevent the overproduction of PGE₂. Therefore, the TriCat Enzyme might be useful in reducing inflammation. This hypothesis requires further testing in vitro and in vivo.

Acknowledgments

This work was supported by NIH Grants (HL56712 and HL79389 (to KHR)). Also, we thank Dr. Richard J. Kulmacz and Dr. Lee-Ho Wang for providing the original wild type cDNAs of human COX-2 and PGIS, respectively. In addition, we thank the Bacu-iovirus/Monoclonal Antibody Facility in Baylor College of Medicine (Houston, TX) for the large-scale expression of the 6His-TriCat Enzyme.

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Fig. 1.

Engineering the TriCat enzyme by linking the C-terminal position of COX-2 to the N-terminal position of PGIS through a transmembrane (TM) linker of 10 amino acid residues. The TriCat enzyme's direct conversion of AA to PGI₂, through its triple catalytic activities, is indicated by the pathway shown.

Fig. 2.

(A) TriCat enzyme expression in Sf-9 cells using a BV expression system. The membrane proteins (20 μg) of ten Sf-9 cell plaques (A–J) transfected with the BV vector containing the TriCat enzyme's cDNA were separated by 7% SDS–PAGE and then Western blot analysis was performed using either (I) anti-PGIS peptide antibody or (II) anti-COX-2 peptide antibody as described [13]. (B) Activity assays for the individual plaques shown in (A) were performed. 20 μg of the membrane protein were incubated with [^{14}C]-AA (10 μM) and the supernatant was analyzed by HPLC-scintillation analysis as described in the Experimental Procedures. The amount of [^{14}C]-6-keto-PGF $_{1\alpha}$ (degraded [^{14}C]-PGI $_2$) produced by each individual plaque of Sf-9 cells was plotted. (C) Western blot analysis for the 'H' plaque of the Sf-9 cells expressing

the 6His-TriCat enzyme at different days after the transfection. (D and E) Analysis of 6His-TriCat enzyme expression by electrophoresis (Coomassie Blue staining, (A) and Western blot analysis with anti-PGIS peptide antibody (B) using the Sf-9 cells from plaque 'H'.

Fig. 3.

Triple catalytic activity assay for the plaque 'H' (upper panel) and the control (transfected with vector alone, lower panel) Sf-9 cells. The crude membrane protein (100 μ g) of the Sf-9 cells were incubated with [¹⁴C]-AA (10 μ M) and then the supernatant was analyzed by HPLC-scintillation analysis as described in Fig. 2. The amount of [¹⁴C]-6-keto-PGF_{1α} (degraded [¹⁴C]-PGI₂) as well as the non-specific side-product, [¹⁴C]-HHT, and the unmetabolized [¹⁴C]-AA are all indicated in the panels.

Fig. 4.

Comparison of the 6His-TriCat enzyme expressed in the Sf-9 cells (1), the wild type TriCat enzyme expressed in the mammalian cell lines, HEK293 (2) and COS-7 (3), and the pcDNA vector (4) expressed in HEK293 cells using activity assays (A) and Western blot analysis (B) as described in Fig. 2.

Fig. 5. HPLC analyses of proteins expressed with different expression systems. The assays were performed under the same conditions as in Fig. 3. $[^{14}\text{C}]$ -AA was added to: (A) HEK293 cells expressing the COX-2 enzyme, (B) Sf-9 cells containing BV-expressed COX-2-10aa-PGIS, or (C) HEK293 cells co-expressing the COX-2 and PGIS enzymes. The major products and by products have also been indicated. Also, the percentage of degraded $[^{14}\text{C}]$ -AA has been determined and is shown as the circled by-products in B and C.

Fig. 6.

Comparison of the kinetic studies for the COX-2-10aa-PGIS prepared from Sf-9 cells (A and C) and the mixture of the individual COX-2 and PGIS (B) co-expressed in HEK293 cells [13]. Different concentrations of [^{14}C]-AA (0.3–60 μM) were added to either the Sf-9 cells or the HEK293 cells in a total reaction volume of 30–100 μL . After a 0.5–5 min incubation, the reaction was terminated and the supernatant was analyzed by HPLC-scintillation analysis as described in the Experimental Procedures. The results were plotted as the average of multiple experiments, where $n = 3$.

Fig. 7.

(I) Effects of the TriCat enzyme on anti-platelet aggregation. The platelet-rich plasma was incubated with 100 μM of AA at 37 $^{\circ}\text{C}$ in the presence of the TriCat enzyme [10 μg , (A)], COX-2 mixed with PGIS [10 μg , (B)] Sf-9 cells only (C), or PBS (D). The addition of the AA to the platelet cells is indicated with an arrow. (II) Dose-dependent inhibition of platelet aggregation by the TriCat enzyme. The platelet aggregation experiments were performed with the addition of increasing amounts of the 6His-COX-2-10aa-PGIS (■) or Sf-9 cells only (●) as described in the Experimental Procedures.

Fig. 8.

(I) Comparison of the effects of anti-platelet aggregation for the BV-expressed COX-2-10aa-PGIS protein (A), the mixture of COX-2 and PGIS expressed in HEK293 cells (B), aspirin (1.3 mg/mL, (C) and untransfected Sf-9 cell protein (D). (II) Comparison of the metabolized AA in the platelet-rich plasma in the absence (A), and presence of the membrane-bound 6His-COX-2-10aa-PGIS (B) or the mixture of the individual COX-2 and PGIS (C). The platelet-rich plasma was incubated with PBS, (A), 10 μ g of membrane-bound 6His-COX-2-10aa-PGIS (B) or the mixture of COX-2 and PGIS [10 μ g total, (C) and then incubated with AA (2 mM) mixed with [¹⁴C]-AA (10 μ M) for 10 min. The supernatant was collected by centrifugation and then profiled by the HPLC-scintillation analyzer (Fig. 3).

Fig. 9.

(A) PAGE analysis of the partially purified 6-His-TriCat enzyme. The following samples: Sf-9 cells expressing the 6-His-TriCat enzyme [crude Sf-9 cell membrane (50 μg , Lane 1)] and the protein extracted by 1% octyl glucoside (50 μg , Lane 2), and then separated by ultracentrifugation (10 μg , Lane 3) and further purified by gel filtration (2 μg , Lane 4), were separated by 7% SDS-PAGE and stained by Coomassie blue. The molecular mass of the 6-His-TriCat enzyme is indicated on the right. (B) Determination of the trip-cat activities for the partially purified 6-His-TriCat enzyme in the conversion of AA to PGI_2 . 5 μg of the purified 6-His TriCat enzyme was incubated with $[^{14}\text{C}]\text{-AA}$ and the products were analyzed by the HPLC-scintillation analyzer as described in Fig. 2 and Fig. 3. The 6-keto- $\text{PGF}_{1\alpha}$ (degraded $[^{14}\text{C}]\text{-PGI}_2$) has been labeled.

Fig. 10.
Phase changes and intramolecular NOEs of ibuprofen (A and B) and U46619 (C and D) induced by COX-2-10aa-PGIS. Green and grey color peaks represent the negative phase (for free ligand) and positive phase (for the ligands bound to protein), respectively. The new intramolecular NOEs of ibuprofen (B) and U46619 (D) induced by the 6-His-TriCat enzyme in comparison with its free state (A and C) are boxed and labeled (B and D).

Table 1Comparison of the K_m values of the COX-2-10aa-PGIS and the individual enzymes

	TriCat enzyme (μM)	COX-2 (μM)	PGIS (μM)
K_m	3.9 (expressed in HEK293 cells)	6.5	13.3
	3.0 (expressed by BV system)	0.9	30