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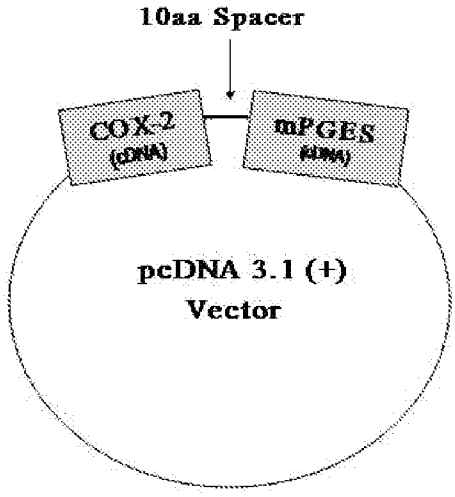
(54) **Title:** HYBRID PROTEIN WITH COX AND PGES ENZYME ACTIVITIES



(57) **Abstract:** A representative hybrid or engineered protein is constructed by linking together human cyclooxygenase (COX) isoform-2 (COX-2) and prostaglandin E_2 synthase (PGES) via a 10-20 amino acid residues of a transmembrane sequence. The single protein molecule is expressed in cells, and adopts the functions of COX and PGES, to continually convert arachidonic acid (AA) into prostaglandin G_2 (catalytic step 1), prostaglandin H_2 (catalytic step 2) and prostaglandin E_2 (PGE_2; catalytic step 3). The COX-2-10aa-mPGES hybrid protein also converts dihomo-γ- linolenic acid (DGLA) into PGE_1.

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HYBRID PROTEIN WITH COX AND PGES ENZYME ACTIVITIES

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BACKGROUND OF THE INVENTION

Field of the Invention

The present invention generally relates to methods and compositions for the prevention and treatment of vascular diseases, and more particularly to such compositions that provide cyclooxygenase (COX) and eicosanoid-synthesizing enzyme functions. Still more particularly the invention relates to the construction and expression of a single linked protein molecule that possesses both the enzyme functions of the native COX and that of PGES.

Description of Related Art

Prostanoids.

The recent discovery that cyclooxygenase (COX) isoform-2 (COX-2) inhibitors may be linked to heart disease has greatly increased the interest in understanding the biology of COX enzymes, which convert a lipid molecule, arachidonic acid (AA), into different prostanoids (part of the eicosanoid family) having diverse and/or opposite biological functions. **Fig. 1** outlines the biosynthesis of prostanoids, comprising prostaglandins and the thromboxane, formed via the COX pathway from AA in three catalytic (tri-catalytic) steps (i-v): Arachidonic acid released from cell membrane phospholipids is converted to the prostaglandin G_2 (PGG_2, catalytic step i), and then to prostaglandin endoperoxide (prostaglandin H_2 (PGH_2)) (catalytic step ii) by COX isoform-1 (COX-1) or COX-2. PGH_2 is further isomerized to biologically active end-products (prostaglandin D_2 (PGD_2), E_2 (PGE_2), F_2 (PGF_2), and I_2 (PGI_2 (prostacyclin) or thromboxane A_2 (TXA_2) by individual synthases (catalytic step iii) in tissue specific manners. Prostanoids act as local hormones in the vicinity of their production site to regulate hemostasis and smooth muscle functions. Unlike the stable level of COX-1 expression, COX-2 expression is inducible and it responds to the stimuli of pro-inflammatory and other pathogenic factors (vi). TXA_2 produced from PGH_2 by TXA_2 synthase (TXAS) has been implicated in various pathophysiological conditions as a proaggregatory and vasoconstricting mediator (vii-viii). PGI_2 is the main AA metabolite in vascular walls and has opposing biological properties to TXA_2, representing the most potent

endogenous vascular protector acting as an inhibitor of platelet aggregation (ix) and a strong vasodilator on vascular beds (x-xiii).

PGE_2 exhibits a variety of biological activities in inflammation (xiv). Aspirin and non-steroidal anti-inflammatory drugs (NSAID) inhibit both COX-1 and COX-2 activities to reduce the production of all prostanoids, which leads to thinning of the blood by reducing TXA_2 production and the suppression of inflammation through decreasing PGE_2 production. The selective COX-2 inhibiting drugs exhibit anti-inflammatory effects similar to aspirin and NSAIDs, but they may also promote strokes and heart attacks by decreasing the production of PGI_2, and increasing the production of TXA_2. This may occur because, when the COX-2 enzyme was specifically inactivated by COX-2 inhibitors, the PGH_2 produced by COX-1 is still available to be converted into other prostanoids such as TXA_2 by TXAS, leading to an increased risk of thrombosis and vasoconstriction (vi).

The function of PGI_2 is primarily mediated by the PGI_2 receptor (IP) on the cell surface. The role of PGI_2 as an endogenous anti-thrombotic and vasodilative agent was confirmed with the experimental data generated in IP receptor-knockout mice. The IP-deficient mice developed without vascular problems in normal situations. However, an increased thrombotic tendency was observed in the IP-deficient mice when endothelial damage was induced. These findings indicate that the anti-thrombotic system mediated by PGI_2 is activated in response to vascular injury to minimize the effects of vascular injury (1, 2, 12). It has been reported that defects in the IP receptor of platelets has pathogenetic significance for developing atherosclerosis at an early age. The evidence was derived from a 10 year-old human diagnosed with an occluded the left popliteal artery who also had a defect of her IP receptor. This defect appears to be genetically determined and to contribute to the development of atherosclerosis (4)

Recently, PGI_2 has also been determined to be a ligand for the nuclear hormone receptor PPAR. In 1990, Issemann & Green reported the discovery of a peroxisome proliferator-activated receptor (PPAR) (5). Since the initial report, three PPAR-isoforms, PPARα, β/δ and γ have been cloned and implicated in the regulation of the expression of genes involved in lipid metabolism. In both skeletal and cardiac muscle cells it has been demonstrated that the metabolic conversion of fatty acids is under controlled by PPARs. PGI_2 and PGI_2 agonists (e.g., carbaprostacyclin and iloprost), can effectively induce DNA binding and transcriptional activation by PPAR. PGI_2, acting as a ligand for PPARδ, induces increases expression of PPARδ in the arterial wall after balloon injury. This observation suggests that PGI_2 effects vasodilation and anti-platelet aggregation through the IP receptor and PPARδ (6). It has also been proposed that PGI_2, as a ligand for PPARδ, induces anti-inflammatory activity in vascular diseases, such as atherosclerosis. The proposed pathway for this anti-inflammatory activity is PGI_2 is bound by PPARδ, leading to the release of BCL-6, a

transcriptional repressor of inflammation, by PPARδ. Release of BCL-6 may cause a decrease expression of inflammatory cytokine genes, reduced inflammation, and a decrease in atherosclerosis. Alternatively, in the absence of PGI_2, BCL-6 may remain bound by PPARδ, resulting in a pro-inflammatory response (7). Recently, PGI_2 production in female reproductive tissues has been documented. PGI_2 is present in the embryo implantation site and human fallopian tubes (8, 9). The presence of PGI_2 at the site of implantation has lead to the proposal that PGI_2 plays an important role in the reproductive process in females, including egg transportation, embryo development, and implantation. A selective increase in the production of PGI_2 with a decrease in TXA_2 production and PGE_2 production is the ideal model in (i) preventing and protecting against vascular diseases including inflammation, thrombosis, atherosclerosis, stroke, and heart attacks, and (ii) benefiting the reproductive process. From aspirin to the more recently developed COX-2 inhibitors, no drugs have yet to achieve this goal.

PGE_2 expression is not only enhanced at sites of inflammation in soft tissues, it also has a variety of other biological activities such as stimulation of new bone formation. In several studies, PGE_2 induced angiogenesis *in vitro* and *in vivo*, and it has been suggested that it mediates wound repair. It has been suggested that PGE_2 has the ability to control cryoprotection and the repair of tissues. Similar to production of PGE_2, prostaglandin E_1 (PGE_1) is produced when PGE synthase converts a different lipid substrate, dihomo-γ-linolenic acid (DGLA), instead of arachidonic acid. PGE_1 has biologic functions that are opposite to PGE_2, similar to PGI_2.

SUMMARY OF PREFERRED EMBODIMENTS

A group of hybrid or engineered proteins, having the general structure COX–linker–PGES, is disclosed herein, along with methods of making and use, and compositions that contain the new hybrid proteins. As discussed and illustrated in the Detailed Description and related drawings which follow, the representative COX-2–10aa–mPGES molecule possesses tricatalytic activity to convert AA to PGE_2. The COX-2–10aa–mPGES molecule also directly converts dihomo-γ-linolenic acid (DGLA) into PGE_1, and, thus, provides a way to specifically increase the biosynthesis of the vascular protector PGE_1. This is believed to be an important development in pharmacology. A representative recombinant 91-kDa engineered protein was constructed by linking together human cyclooxygenase (COX) isoform-2 (COX-2) and microsomal prostaglandin E_2 synthase (mPGES) via 10 amino acid residues of a transmembrane sequence. This engineered protein was successfully expressed in human embryonic kidney cells (HEK293) and adopted the functions of COX-2 and mPGES, which was able to continually convert arachidonic acid (AA) into prostaglandin G2 (catalytic step 1), prostaglandin H2 (catalytic step 2) and prostacyclin (PGI2; catalytic step 3).

Accordingly, certain embodiments of the present invention provide a new COX-2–linker–mPGES protein, and demonstrate that multiple catalytic activities of enzymes can be configured within a single protein molecule if the correct protein configuration is achieved.

In accordance with certain embodiments of the present invention, methods are provided for combining the enzymatic functions of COX and PGES in a single "hybrid" peptide molecule.

In accordance with certain embodiments of the present invention, a new generation of cDNA for COX gene therapy is provided, in which the cDNA encodes an above-described hybrid molecule. In accordance with other embodiments, a COX–linker–PGES protein is used as a therapeutic reagent. For example, in some embodiments it will be useful to provide an opposite biological function to that of PGE_2, especially with respect to vascular effects. An instant increase in local production of PGE_1 is expected through local injection of the engineered protein. In some embodiments, the hybrid protein acts on a lipid molecule other than arachidonic acid, dihomo-γ-linolenic acid (DGLA), to produce PGE_1 which has activity similar to that of PGI_2.

In accordance with certain other embodiments of the present invention, a therapeutically useful hybrid protein is provided that comprises a single molecule containing COX–linker–mPGES. The linker sequence is disposed between and directly connects the COX amino acid sequence to the mPGES amino acid sequence. In certain embodiments, the linker amino acid sequence is capable of functioning as a transmembrane linker in a cell so that the folding ability and functions of both enzymes are substantially unaltered compared to the folding ability and function of the respective native enzymes.

In certain embodiments, the linker sequence is about 10 to 22 amino acids long. In certain embodiments, the linker sequence is His-Ala-Ile-Met-Gly-Val-Ala-Phe-Thr-Trp (SEQ ID NO. 1) or His-Ala-Ile-Met-Gly-Val-Ala-Phe-Thr-Trp-Val-Met-Ala-Leu-Ala-Cys-Ala-Ala-Pro-Pro-Leu-Val (SEQ ID NO. 2). In certain embodiments, the linker sequence is residues 1-11, 1-12, 1-13, 1-14, 1-15, 1-16, 1-17, 1-18, 1-19, 1-20 or 1-21 of SEQ ID NO. 2. In certain embodiments, the linker peptide provides approximately 10 Å separation between the catalytic sites of the COX and the mPGES enzyme. The connected enzymes are preferably capable of substantially normal folding and enzymatic activity compared to the native folding and enzymatic activity of the native COX and mPGES enzymes. In accordance with certain embodiments of the present invention, a pharmaceutical composition is provided which comprises an above-described hybrid protein together with a pharmaceutically acceptable carrier.

Certain embodiments of the present invention provide an isolated DNA sequence encoding: a cyclooxygenase (COX), a transmembrane linker peptide, and a microsomal prostaglandin E_2-synthesizing enzyme (mPGES). In some embodiments, the cyclooxygenase is a cyclooxygenase isoform-1 (COX-1). In some embodiments, the cyclooxygenase is a

cyclooxygenase isoform-2 (COX-2). Certain embodiments of the present invention provide a vector comprising an above-described DNA sequence. In some embodiments, the vector is an expression vector.

Some embodiments of the present invention provide a host cell containing an expressible DNA sequence encoding an above-described hybrid protein.

Another embodiment of the present invention provides a process for producing a hybrid protein that comprises a cyclooxygenase, a transmembrane linker, and a prostacyclin synthase. The process comprises culturing the above-described host cell under conditions suitable for expression of the DNA sequence encoding the hybrid protein molecule, and then recovering the biologically active hybrid protein molecule comprising a cyclooxygenase, a transmembrane linker, and a prostaglandin E_2 synthase. Preferably the hybrid protein has the general structure COX-2–linker–mPGES, wherein the linker is the 10 aa sequence of SEQ ID NO. 1. In some embodiments, the process comprises, prior to the culturing step, transfecting the host cell with a vector comprising the DNA sequence. These and other embodiments, features and advantages will be apparent with reference to the following detailed description and accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is an outline showing the biosynthesis of prostanoids through the coordination of the COX enzymes and their downstream synthases.

FIG. 2 is an illustration showing the engineered cDNA plasmids with single proteins containing a COX-2 sequence, a PGIS sequence, and a transmembrane linker sequence.

FIGS. 3A-B depict the western blot analysis of an overexpressed COX-2–linker–PGIS proteins in COS-7 cells (A) or HEK293 cells (B).

FIGS. 4A-B depict immunofluorescence micrographs of HEK293 cells. A: anti-human PGIS antibody. B: anti-human COX-2 antibody.

FIGS. 5A-B are graphs showing the tri-catalytic activities of the linked proteins directly converting AA to PGI2 using a High Performance Liquid Chromatography (HPLC) method, in which a: COX-2–22aa–PGIS. b: COX-2–10aa–PGIS. c: PGIS–10aa–COX-2. d: COX-2 and PGIS. e: untransfected cells.

FIGS. 6A-B are graphs showing the tri-catalytic activities of the linked proteins directly converting AA to PGI2 using Enzyme Immunoassay (EIA) analysis. (A) Expressed in COS-7. (B) Expressed in HEK293 cells.

FIG. 7 is a pair of graphs showing a comparison of the tri-catalytic activities of an engineered COX-2–linker–PGIS protein in the intact cells (A) compared to the membrane preparations (B).

FIG. 8 is a group of graphs showing the effects of a COX-2 inhibitor on the tri-catalytic activities of a representative COX-2–linker–PGIS.

FIG. 9 is a graph showing the enzyme kinetic properties of the COX-2–linker–PGIS tri-catalytic properties.

FIG. 10 is a graph showing the time-course of the conversion of AA to PGI2 by the COX-2–linker–PGIS proteins.

FIG. 11 is a flow diagram illustrating the subcloning of cDNA of the engineered COX-2–10aa–PGIS into the pcDNA3.1(+) vector using Hind III and Bgl II sites (A). Section B depicts agarose gel separation of the digested plasmid containing the COX-2–10aa–PGIS.

FIG. 12 depicts an engineered cDNA plasmid containing the COX-2 linked to mPGES sequence.

FIG. 13 depicts a Western Blot analysis of the over-expressed recombinant proteins in HEK293 cells. Lane 1: transfection with COX-2–10aa–mPGES cDNA plasmid. Lane 2: co-transfection with COX-2 cDNA plasmid and human mPGES cDNA plasmid. Lane 3: (negative control) transfection with purified cDNA 3.1(+) plasmid.

FIG. 14 depict immunofluorescence micrographs of HEK293 cells. Column A: Anti-COX-2 peptide antibody. Column B: Anti-mPGES antibody. Micrographs 1 and 2: transfected with plasmid containing COX-2–10aa–mPGES cDNA. Micrograph 3: transfected with plasmid containing COX-2 cDNA. Micrograph 4: transfected with plasmid containing mPGES cDNA. Micrographs 5 and 6: transfected with purified cDNA vector.

FIG. 15 depict HPLC analysis determining the tri-catalytic activities of the recombinant proteins directly converting AA to PGE_2 in HEK293 cells. The cells were transfected with the recombinant cDNAs of COX-2–10aa–mPGES (A), COX-2 and mPGES (B), COX-2 only (C), mPGES only (D), or pcDNA 3.1 vector only (E). Untransfected cells (control): (F). Addition of a COX-2 inhibitor (NS-398, 10 μM) to cells transfected with COX-2-10aa-mPGES (G).

FIG. 16 is a bar graph showing tri-catalytic activities of the recombinant proteins directly converting AA to PGE_2 using Enzyme Immunoassay (EIA). Bar 1: cells transfected with plasmid containing cDNA of COX-2–10aa–mPGES. Bar 2: cells transfected with plasmid containing cDNA of COX-2 and transfected with plasmid containing cDNA of mPGES. Bar 3: cells transfected with purified cDNA vector alone.

FIGS. 17A-B are graphs showing that the tri-catalytic activities of the recombinant proteins direct convert DGLA to PGE_1, as determined using an HPLC method for HEK293 cells. A: Cells transfected with COX-2–10aa–mPGES recombinant cDNA. B: Cells transfected with COX-2 recombinant cDNA and recombinant mPGES cDNA.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Recent studies of the structure and function relationship of COX enzymes and PGIS have advanced our knowledge of the molecular mechanisms involved in the biosynthesis of PGI_2 in native cells. Crystallographic studies of detergent-solubilized COX-1 and COX-2 suggest that the catalytic domains of the proteins lie on the luminal side of the endoplasmic reticulum (ER) and are anchored to the ER membrane by the hydrophobic side chains of the amphipathic helices A-D. These hydrophobic side chains of the putative membrane anchor domains also form an entrance to the substrate-binding channel and potentially form an initial docking site for the lipid substrate, AA (13-14). Recent progress in the topology and structural studies of human PGIS and TXAS have led to the proposal of models in which PGIS and TXAS have catalytic domains on the cytoplasmic side of the ER, opposite the orientation of COXs (15-17). In this configuration, the substrate channels of all the three enzymes, COX, PGIS and TXAS, open at or near the ER membrane surface. The coordination between COXs and PGIS or TXAS in the biosynthesis of TXA_2 and PGI_2 may be facilitated by the enzyme's anchoring in the lipid membrane (18). The physical distances between COXs and PGIS are very small. It was hypothesized that it is possible to create a single protein molecule containing COX and PGIS sequences with minimum alteration of both enzymes' folding and membrane topologies by extending the N-terminal membrane anchor domain of PGIS using a transmembrane sequence linked to the COX-1 or COX-2, which then adopts the functions of both enzymes of COX and PGIS. In this case, AA can be directly converted into the vascular protector, PGI_2, with decreases of the unwanted PGE_2 and TXA_2 productions.

The studies described herein are the first to demonstrate that a single, linked protein molecule can perform triple enzyme catalytic functions. One highly desirable feature of certain embodiments of the invention is that the linked protein allows for the direct synthesis of the potent vascular protector, PGI_2, from AA with a high efficiency, which may be used to prevent and rescue patients from vascular disease, including strokes, heart attacks, thrombosis, and ischemia through specifically increasing PGI_2 production. Gene delivery of the engineered cDNA and/or giving the engineered protein to tissues *in vivo* is one means for accomplishing these benefits. The initial discovery with respect to PGIS has been confirmed with PGES, providing further evidence that the COX enzymes can be linked with other downstream eicosanoid-synthesizing (ES) enzymes, thereby generating still other linked proteins, to specifically regulate the biosynthesis of eicosanoids, which play diverse and potent biological functions in human.

Example 1

Preparation of Linked Protein

To test this hypothesis, a linker with 10 (His-Ala-Ile-Met-Gly-Val-Ala-Phe-Thr-Trp (10aa)) or 22 (His-Ala-Ile-Met-Gly-Val-Ala-Phe-Thr-Trp-Val-Met-Ala-Leu-Ala-Cys-Ala-Ala-Pro-Pro-Leu-Val (22aa)) residues of the structurally defined helical transmembrane domain of bovine rhodopsin were used to configure the engineered cDNA containing the COX-2 and PGIS sequences. The sequence begins at the N-terminus of COX-2, which is linked to either DNA encoding 10aa (COX-2–10aa–PGIS) or DNA encoding 22aa (COX-2–22aa–PGIS), and then these are linked to the N-terminus of PGIS that ends with the C-terminus of PGIS. In contrast, an engineered cDNA containing reversed sequences from PGIS, DNA encoding 10aa to COX-$_2$, were also prepared as a control (PGIS–10aa–COX-2 (Fig. 2).

Engineered cDNA plasmids with single genes encoding the COX-2 or PGIS sequences. COX-2 linked to PGIS, and PGIS linked to COX-2 through the 10aa or 22aa sequence were generated by PCR approach (19) and subcloning procedures provided by the vector company (Invitrogen). Briefly, the corresponding cDNA sequences were isolated from the pSG5 or pcDNA3.1 vector containing human COX-2 or PGIS by PCR using the primers containing the DNA encoding 10aa or DNA encoding 22aa and KpnI or Bam HI cutting sites at both end (the 5' end of the anti-sense primer was connected with the DNA sequences of the designed linker). The resulting cDNA segment was cut with the corresponding restriction enzymes and ligated into the corresponding sites at the pcDNA3.1 vector.

Example 2

Detection of Linked Protein

The cDNAs of the engineered COX-2–10aa–PGIS, COX-2–22aa–PGIS, and PGIS–10aa–COX-2 were successfully cloned into a pcDNA3.1 vector containing a cytomegalovirus early promoter using a PCR (polymerase chain reaction) cloning approach (Fig. 2). In Fig. 2, engineered cDNA plasmids with single proteins containing COX-2 and PGIS sequences are shown. COX-2 linked to PGIS, and PGIS linked to COX-2 through the 10aa or 22aa sequence were generated by PCR approach (19) and subcloning procedures provided by the vector company (Invitrogen). Briefly, the corresponding cDNA sequences were isolated from the pSG5 or pcDNA3.1 vector containing human COX-2 or PGIS by PCR using the primers containing the 10aa or 22aa and KpnI or Bam HI cutting sites at both ends (the 5' end of the anti-sense primer was connected with the DNA sequences of the designed linker). The resulting cDNA segment was cut with the corresponding restriction enzymes and ligated into the corresponding sites at the pcDNA3.1 vector. The correct sequences were confirmed by DNA sequencing and endonuclease digestion analyses. The expression of the recombinant proteins was tested in COS-7 cell line (monkey

epithelial cells) and HEK293 cell line (human embryonic kidney 293 cells) by a transient protein expression approach using Lipofectamin200 (Invitrogen). COX-2–10aa–PGIS, and COX-2–22aa–PGIS were successfully overexpressed in the both cell lines having a molecular weight of approximately 130 kDa as demonstrated by Western Blot analysis (Figs. 3A,B). In contrast, the expressed PGIS–10aa–COX-2 protein was undetected by Western Blot indicating that it may have been degraded due to the lack of a correct protein folding. Figs. 3A,B show a Western blot analysis of the overexpressed recombinant proteins in COS-7 (A) or HEK293 (B) cells. The procedures were described previously (15-17). Briefly, COS-7 or HEK293 cells were grown for 24 hours to 90-95% confluent and then transfected with a purified cDNA plasmid (24 μg/dish (100 mm)) by the Lipofectamin™2000 method (20) following the manufacturer's instructions (Invitrogen). For the co-transfection, the cells were transfected with 12 μg of human COX-2 cDNA plasmid and 12 μg of human PGIS cDNA plasmid. Approximately 48 hours after transfection, the cells were harvested for Western blot analysis. 20 μg of the proteins of the cells transfected with the cDNA(s) of COX-2–10aa–PGIS (lane 1), COX-2–22aa–PGIS (lane 2), PGIS–10aa–COX-2 (lane 3) or untransfected cells (lane 4) were subjected to SDS-PAGE and then transferred onto a nitrocellulose membrane. The membrane was probed with rabbit anti-PGIS peptide antibody (A) or anti-COX-2 peptide antibody (B) and then stained with horseradish peroxidase-labeled goat-anti rabbit antibody. Molecular weight standards were run, and the lines on the left and right show the positions of the these standards. The engineered proteins are indicated with arrows.

Example 3

Localization of Linked Protein to Endoplasmic Reticulum Membrane

To test whether the expressed COX-2–10aa–PGIS and COX-2–22aa–PGIS could anchor to the ER membrane, adopting similar membrane topologies as the native COX-2 and PGIS, fluorescence immunostaining was used to localize the subcellular distribution of the engineered enzymes overexpressed in COS-7 cells. A similar pattern of ER staining was clearly observed in the COS-7 cells expressing the engineered proteins compared to the cells co-expressing the individual COX-2 and PGIS using either anti-human PGIS (Fig. 4A) or anti-human COX-2 (Fig. 4B) antibodies. Low-level expression of PGIS–10aa–COX-2 was also observed in the transfected cells, (Figs. 4A,B). The correct pattern of ER staining indicates that the engineered COX-2–10aa–PGIS and COX-2–22aa–PGIS have correct folding and ER membrane anchoring functions and are more suitable for enzymatic activity compared to the PGIS–10aa–COX-2 engineered protein.

In Figs. 4A,B, immunofluorescence micrographs of HEK293 cells are shown. The general procedures for the indirect immunostaining were described previously (15-16, 21). In brief, the cells were grown on cover-slides and transfected with cDNA plasmid(s) as described in Fig 3. The cells were generally permeabilized by saponin, and then incubated with the affinity-

purified rabbit anti-PGIS peptide antibody (A) (16) or mouse anti-COX-2 antibody (B). The bound antibodies were incubated with FITC-labeled goat-anti rabbit IgG (A) or Rhodomin-labeled rabbit anti-mouse IgG (B). The FITC-labeled goat-anti rabbit IgG (A) or Rhodomin-labeled rabbit anti-mouse IgG were examined by fluorescence microscopy (16).

Example 4

Detection of PGI_2

Evidence for the conversion of AA into the vascular protector PGI_2 by the tri-catalytic reactions in a single protein of COX-2–10aa–PGIS or COX-2–22aa–PGIS were given by two independent assays, High Performance Liquid Chromatography (HPLC) analysis (Fig. 5A and Fig. 5B), and Enzyme Immunoassay (EIA, Figs. 6A,B) using AA as a substrate for the cells overexpressing the engineered proteins. The tri-catalytic activities in the engineered single COX-2–10aa–PGIS or COX-2–22aa–PGIS proteins were identical to the reactions mediated by co-expression of COX-2 and PGIS in the cells (Figs. 5A and B, and 6A,B). It should be noted that the synthesized PGI_2 was unstable and quickly degraded into a stable 6-keto-$PGF_{1\alpha}$. Thus, the level of 6-keto-$PGF_{1\alpha}$ shown in the HPLC and EIA represents the amounts of PGI_2 produced by the enzymatic reactions (Figs. 5A and B, 6A-B). COX-2–10aa–PGIS and COX-2–22aa–PGIS expressed in the intact cells exhibited very similar tri-catalytic activities in the direct conversion of AA to PGI_2. The similar catalytic activities were also observed for the membrane protein preparations as compared to the intact cells (Fig. 7, A and B). Thus, the COX-2–10aa–PGIS was used as an example of enzyme properties of COX-2–10aa–PGIS and COX-2–22aa–PGIS.

Determination of the tri-catalytic activities of the recombinant proteins directly converting AA to PGI_2 using a HPLC method. The cells (~0.1 x 10^6) transfected with the recombinant cDNA(s) of COX-2–22aa–PGIS (A), COX-2–10aa–PGIS (B), PGIS–10aa–COX-2 (C) and COX-2 and PGIS (D) as described in Figs. 3A,B, or the untransfected cells (E) were washed three times, suspended in 0.01 M phosphate buffer, pH 7.2 containing 0.15% NaCl (PBS) and then incubated with [^{14}C]-AA (10 μM) in a total volume of 0.1 ml. After five min., the reaction was terminated by addition of 0.5 ml of 0.1% acetic acid containing 30% acetonitrile (buffer A), and centrifuged at 12,000 rpm for 10 min. The supernatant was separated by HPLC on a C18 column (4.5 x 250 mm) using buffer A with a gradient of 30-80% acetonitrile. The [^{14}C]-AA metabolites were determined by a liquid scintillation analyzer built into the HPLC system. The retention time of [^{14}C]-6-keto-$PGF_{1\alpha}$ and [^{14}C]-AA were calibrated by standards under the same conditions. The amount of 6-keto- $PGF_{1\alpha}$ produced represents the amount of PGI_2 produced.

Determination of the tri-catalytic activities of the recombinant proteins directly converting AA to PGI_2 using Enzyme Immunoassay (EIA). After the enzyme reactions as

described in Figs. 5A and B, the reaction mixtures (1. Untransfected cells, 2. COX-2–22aa–PGIS, 3. COX-2–10aa–PGIS, 4. PGIS–10aa–COX-2, and 5. Co-expressed COX-2 and PGIS) were diluted 100 times with PBS containing 0.1% BSA, and then used for quantitative determination of 6-keto-$PGF_{1\alpha}$ using an EIA kit following the instructions of the manufacture (Cayman Chemical, Ann Arbor, MI).

Comparison of the tri-catalytic activities of the engineered COX-2–10aa–PGIS in the cells and in the membrane preparation. The tri-catalytic activities of COX-2–10aa–PGIS expressed in COS-7 cells were determined using the intact cells (A) as described in Figs. 5A and B. The same amount of cells was homogenized and the total membrane protein was collected by ultracentrifugation and used for the activity assay (B). The amount of 6-keto-$PGF_{1\alpha}$ represents the amount of PGI_2 produced.

In Figs. 5A and B the determination of the tri-catalytic activities of the recombinant proteins directly converting AA to PGI_2 using a HPLC method is shown. The cells (~0.1 x 10^6) transfected with the recombinant cDNA(s) of COX-2–22aa–PGIS (A), COX-2–10aa–PGIS (B), PGIS–10aa–COX-2 (C) and COX-2 and PGIS (D) as described in Figs. 3A,B or the untransfected cells (E) were washed three times, suspended in 0.01 M phosphate buffer, pH 7.2 containing 0.15% NaCl (PBS) and then incubated with [^{14}C]-AA (10 μM) in a total volume of 0.1 ml. After five minimums, the reaction was terminated by addition of 0.5 ml of 0.1% acetic acid containing 30% acetonitrile (buffer A), and centrifuged at 12,000 rpm for 10 min. The supernatant was separated by HPLC on a C18 column (4.5 x 250 mm) using buffer A with a gradient of 30-80% acetonitrile. The [^{14}C]-AA metabolizes were determined by a liquid scintillation analyzer built in the HPLC system. The retention time of [^{14}C]-6-keto-$PGF_{1\alpha}$ and [^{14}C]-AA were calibrated by standards under the same conditions. The amount of 6-keto- $PGF_{1\alpha}$ produced represents the amount of PGI_2 produced.

In Figs. 6A-B, the determination of the tri-catalytic activities of the recombinant proteins directly converting AA to PGI_2 using Enzyme Immunoassay (EIA) is shown. After the enzyme reactions as described in Figs. 5A and B, the reaction mixtures (1. Untransfected cells, 2. COX-2–22aa–PGIS, 3. COX-2–10aa–PGIS, 4. PGIS–10aa–COX-2, and 5. Co-expressed COX-2 and PGIS were diluted 100 times with PBS containing 0.1% BSA, and then used for quantitative determination of 6-keto-$PGF_{1\alpha}$ using an EIA kit followed the instructions of the manufacture (Cayman Chemical, Ann Arbor, MI).

Fig. 7 shows a comparison of the tri-catalytic activities of the engineered COX-2–10aa–PGIS in the cells and in the membrane preparation. The tri-catalytic activities of COX-2–10aa–PGIS expressed in COS-7 cells were determined using the intact cells (A) as described in Figs. 5A and B. The same amount of cells was homogenized and the total membrane protein was

collected by ultracentrifugation and used for the activity assay (B). The amount of 6-keto- $PGF_{1\alpha}$ produced represents the amount of PGI_2 produced.

Example 5

Biological Activity of Linked Protein

The biological activity of the COX-2–10aa–PGIS molecule directly converting [^{14}C]-AA to [^{14}C]-PGI_2 was further confirmed by an inhibition assay using a COX-2 inhibitor, NS-398, and a PGIS inhibitor, U46619 (Fig. 8).

The effects of the COX-2 inhibitor on the tri-catalytic activities of COX-2–10aa–PGIS are shown in Fig. 8, which demonstrates the conversion of AA to PGI_2 by the COX-2–10aa–PGIS overexpressed in COS-7 (A, B and E) and in HEK293 (C and D) cells in the absence (A and C) and presence (B and D) of a COX-2 inhibitor, NS-398 (60 μM), or in the presence of a PGIS inhibitor, U46619 (60 μM, E), using the HPLC method as described in Figs. 5A and B.

Fig. 9 shows the enzyme kinetic properties of the COX-2–10aa–PGIS with the tri-catalytic activities. Different concentrations of AA was added to the membrane preparations of the COS-7 cells overexpressed COX-2–10aa–PGIS protein (~20 μg), after the incubations for five minutes, the degraded PGI_2 product, 6-keto-$PGF_{1\alpha}$,was determined by EIA method as described in Figs. 6A-B. The membrane preparation of the untransfected COS-7 cells was used as controls. The detailed kinetic studies revealed that the engineered COX-2–10aa–PGIS has a Km value (~3.2 μM, Fig. 9) similar to the reported Km values for the individual COX-2 and PGIS enzymes. These data demonstrate that the engineered COX-2–linker–PGIS adopts the full enzymatic activities of both native COX-2 and PGIS. The tri-catalytic activities in converting AA to PGG_2 and ultimately to PGH_2 and to PGI_2 can be completed by the engineered single protein.

Enzyme kinetic properties of the COX-2–10aa–PGIS with the tri-catalytic activities. Different concentrations of AA was added to the membrane preparations of the COS-7 cells overexpressed COX-2–10aa–PGIS protein (~20 μg), after the incubations for five minimums, the degraded PGI_2 product, 6-keto-$PGF_{1\alpha}$, was determined by EIA method as described in Figs. 6A-B. The membrane preparation of the untransfected COS-7 cells was used as controls.

Additionally, in time course studies, the tri-catalytic turn-over rate of the COX-2–10aa–PGIS in the first 0.5-1 min reaction was faster than the reactions using the two enzymes of COX-2 and PGIS co-expressed (Fig. 10). Time-course of the conversion of AA to PGI_2 by the recombinant proteins is shown in Fig. 10. Referring still to Fig. 10, the conversion of AA to PGI_2 by the COX–10aa–PGIS (•) or the co-expressed COX-2 and PGIS in the HEK293 cells (■) were performed with different reaction times using the EIA method as described in Fig. 6A-B. The amounts of the produced PGI_2 (6-keto-$PGF_{1\alpha}$) at the different reaction times were plotted. The untransfected cells were used as controls.

The results demonstrate that the engineered molecules can compete with the endogenous COX-downstream synthases to convert COX-generated PGH_2 to PGI_2. Thus, the engineered COX-2–linker–PGIS not only adopts the COX and PGIS activities but also increases the selectivity of converting AA to PGI_2. This is of particular importance in pathophysiological conditions, in which quick conversion of AA or PGH_2 to PGI_2 will reduce the substrate available to other COX-downstream synthases such as TXAS and PGES. Incorporating either of the engineered proteins *in vivo* will cause a decrease in the biosynthesis of TXA_2 and PGE_2 in the cells, which will be important for preventing and reversing thrombosis, hypertension, and ischemic tissues resulting from TXA_2 and for the reduction of vascular inflammation stimulated by PGE_2. The cDNA of the COX-2–linker–PGIS may be used as a gene therapy reagent to prevent and treat the thrombosis associated with strokes and heart attacks. In addition, the biologically active COX-2–linker–PGIS protein may be used as a reagent by injection into tissues to rapidly synthesize PGI_2 *in vivo*.

The COX-2–linker–PGIS proteins offer many advantages, including its unique protein design has demonstrated that multiple catalytic activities of enzymes can be configured within a single protein molecule if the correct protein configuration was achieved (10). It is demonstrated that the new hybrid protein, with tri-catalytic activity, not only possesses the individual enzymes' activities, but has a faster turnover rate as compared to a mixture of the parent enzymes (11). Since COX-1 shares similar molecular features with COX-2, the method used for preparation of COX-2–linker–PGIS is also suitable for general preparation of the COX-1–linker–PGIS molecule, or any other prostaglandin synthase (12). The methods and information used to combine the enzymatic functions of COX-2 and PGIS may also be used to create single "hybrid" peptide molecules containing COX-1 or COX-2 linked with any downstream synthase, such as COX–linker–PGES, COX–linker–PGDS, and COX–linker–PGFS (13).

Successfully creating the functional COX-2–linker–PGIS peptide has settled several important controversies in COX biology For decades it was unknown whether delivery of PGH_2 from COXs to their downstream synthases required a carrier protein (10). As demonstrated herein, the process of PGH_2 moving from COX-2 to PGIS can be achieved within a single molecule and it is suggested that the process does not need a carrier protein. The concept that the enzymatic activities of COXs occur under the conditions of the defined dimerization structures has been generally adopted because the studies of crystal structures for detergent solubilized COX-1 and COX-2 revealed that the active enzymes were in dimer forms (11) . This concept is not fully supported by current studies because it is highly probable that the engineered protein has different dimerization features relative to the native COX enzymes. For decades, the exact physical distances between COXs and their downstream synthases in the cells during the biosynthesis of prostanoids were unknown (12). The present studies clearly demonstrate that the distance between the two enzymes

must be very close because the turn over rate of the isomerization of PGH_2 to PGI_2 in the cells that co-expressed COX-2 and PGIS is very similar to the single molecule of COX-2–10aa–PGIS which has an approximately 10 Å separation between the catalytic sites of COX-2 and PGIS (Fig. 10).

Example 6

Subcloning the cDNA Encoding the Engineered COX-2–10aa–PGIS Gene into Adeno-associated Virus Vector

Adeno-associated virus (AAV) vector is one of the best viral vectors for gene transfer into cells and tissues *in vitro* and *in vivo*. In order to establish an effective gene transfer system for the COX-2–10aa–PGIS used for ischemic therapy in vivo, the cDNA of the engineered COX-2–10aa–PGIS in the pcDNA3.1(+) vector was subcloned into pAAV-MCS vector from AAV Helper-Free system (Stratagene, Cedar Creek, TX) using Hind III and Bgl II sites (pAAV-COX-2–10aa–PGIS, **Fig. 11**, section **A**). The successful cloning was confirmed by endonuclease digestion analyses (**Fig. 11**, section **B**), and the correct sequence was further confirmed by DNA sequencing.

Preparation of the primary pAAV–COX-2–10aa–PGIS viral stock

The viral stock of pAAV–COX-2–10aa–PGIS was prepared following the manufacturer's instructions (Stratagene). Briefly, the AAV-293 cells were plated in 100 mm tissue culture plates with 10 ml of DMEM growth medium 48 hours prior to transfection until they were approximately 70-80% confluent. 10 μg of pAAV-COX-2–10aa–PGIS was co-transfected with two other plasmids (10 μg) provided from the system: pAAV-RC and pHelper. 1 ml of 0.3M $CaCl_2$ was added to the three plasmids and mixed gently. The mixture was then added to 1 ml 2 × HBS drop wise and mixed gently. The DNA/$CaCl_2$/HBS suspension was immediately applied to the plate of cells drop wise and swirling gently to distribute the suspension evenly in the medium. The plates were incubated at 37°C for 6 hours and the medium was then replaced with 10 ml fresh DMEM growth medium. After incubating for an additional 66-72 hours at 37°C, the cells with DMEM growth medium were scraped into the tube and subjected to four rounds of freeze/thaw by alternation of the tube between the dry ice-ethanol bath and the 37°C water bath (with brief vortexing after each thaw). The cellular debris was collected by centrifugation at 10,000 × g for 10 min and the supernatant (primary pAAV-COX-2–10aa–PGIS virus stock) was stored at -80°C. The virus stock was then concentrated and purified using the AAV-Pure™ Maxi AAV purification kits (Biovintage, San Diego, CA). The pAAV-COX-2–10aa–PGIS viral stock is readily available in the inventor's laboratory to infect cells/tissues *in vitro* and *in vivo*.

In present day gene therapy, introducing the COX gene alone into cells may not increase the biosynthesis of PGI_2 due to the limited amount of endogenous PGIS in the cells. Introducing the PGIS gene alone into the cell may also not increase PGI_2 synthesis due to the limited amount of endogenous COXs in the cells. Co-introducing un-linked COX and PGIS genes into cells is reasonably anticipated to increase the difficulty of gene delivery and would not provide specific

control of PGI_2 production due to the competition of the other downstream synthases, which share PGH_2 as their substrate. For example, it is particularly difficult to avoid the over-producing of PGE_2 when large amount of the microsomal PGES-1 is induced by the tissue injuries during the gene delivery. The presently engineered single COX-2–linker–PGIS eliminates the step of PGH_2 movement from the COX protein to the PGIS protein, which are normally in separate locations, leading to increased PGI_2 production and limiting PGH_2 availability for TXA_2 and PGE_2 production. Thus, the hybrid molecules and compositions offer a new generation of cDNA for COX gene therapy.

Alternatively, the COX–linker–PGIS protein may be used as a therapeutic reagent to instantly increase PGI_2 production locally through injection of the engineered protein. It is particularly interesting that COX-2 inhibitors inhibit COX-2 activity but not the COX-1 activity. Thus, introduction of the COX-1–linker–PGIS to vascular systems may be used to overcome the damage of the vascular functions caused by COX-2 inhibitors. Given the importance of PGI_2 in vascular diseases and thrombosis, the presently engineered COX–linker–PGIS will be a useful therapeutic molecule *in vivo*.

Example 7.

Design of a Recombinant Protein with Triple Catalytic Activities Directly Converting Arachidonic Acid (AA) into Prostaglandin E_2

Based on the topological arrangement in the ER membrane of the COX and PGIS proteins, a method of linking active human COX-2 and PGIS together was devised and examined, as described in the foregoing examples. It was proposed that the above-described methods utilizing PGIS can also be applied to engineer other proteins linking COX-1 or COX-2 with its other downstream synthases. To test this hypothesis, the cDNA of human COX-2 was linked with the cDNA of microsomal prostaglandin E_2 synthase (mPGES), one of the other COX downstream synthases, by a DNA sequence encoding the transmembrane domain (TM) of 10aa previously used for linking COX-2 and PGIS as described above using the PCR approach for engineering the COX-2-10aa-PGIS protein. In this case, the sequence begins at the N-terminus of COX-2, which is linked to the 10aa linker and then linked to the N-terminus of mPGES that ends with the C-terminus of mPGES. Thereby, a new cDNA encoding a new single protein, COX-2–10aa–mPGES was generated (Fig. 12). The new cDNA was subcloned into pcDNA3.1(+) vector (pcDNA COX-2–10aa–mPGES (Fig. 12). The subcloning procedure was provided by the vector company (Invitrogen). Briefly, the plasmid of COX-2–10aa–PGIS in pcDNA3.1(+) vector was previously generated with Bam HI cutting sites at both ends of PGIS. The cDNA sequence of human mPGES was isolated 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 already in the pcDNA 3.1(+) vector.

Expression of the Engineered COX-2–10aa–mPGES Protein in HEK Cells

Referring to Fig. 13, the expression of the recombinant COX-2–10aa–mPGES protein was tested in a HEK293 cell line by a transient protein expression approach using Lipofectamine™2000 (Invitrogen). The cells were grown for 24 hours on 100mm 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 cDNA plasmid. For the COX-2–10aa–mPGES (Lane 1) and pcDNA 3.1(+) (negative control, Lane 3), 24 μg of each plasmid were used for each individual transfection. Approximately 48 hours after transfection, the cells were harvested and used for Western blot analysis. Then, 25 μg of protein was applied to each lane and subjected to 7% SDS-PAGE separation, and then transferred to a nitrocellulose membrane. The membrane was then probed with a mixture of rabbit anti-mPGES 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). In Fig. 13, the numbers on the left represent the molecular weight (in kDa) of the proteins described in parentheses. COX-2–10aa–mPGES was successfully overexpressed in this cell line by showing a molecular weight of approximately 91 kDa in Western Blot analysis (Lane 1). In contrast, the co-expressed COX-2 and mPGES enzymes appear at their designated locations of 72kDa and 17kDa, respectively (Lanes 2 and 3).

Referring now to Fig. 14, to test whether the expressed COX-2–10aa–PGES could anchor to the ER membrane, adopting membrane topologies similar to those of the native COX-2 and mPGES, fluorescence immunostaining was used to localize the sub-cellular distribution of the engineered enzymes overexpressed in HEK293 cells. The general procedures for the indirect immunostaining were described previously. In brief, the cells were grown on cover slides and transfected with the plasmid containing the cDNA of COX-2-10aa-mPGES (1 and 2), COX-2 (3), mPGES (4), or pcDNA vector (5) and (6), as indicated in the immunofluorescence micrographs of HEK293 cells shown in Fig. 14. 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. A similar pattern of ER staining was clearly observed in the HEK293 cells expressing the engineered COX-2–10aa–mPGES protein compared to the cells expressing the individual COX-2 and mPGES using either anti-human COX-2 (Fig. 14A) or anti-human mPGES (Fig. 14B) antibodies. The results

indicated that the engineered COX-2–10aa–mPGES has ER membrane anchoring functions similar to those of the native COX-2 and mPGES enzymes.

Directly Converting AA into PGE_2, by the Engineered COX-2-10aa-mPGES with Triple Catalytic Activities

Evidence of the conversion of AA into PGE_2 by the single protein COX-2–10aa–mPGES was indicated by two independent assays: HPLC analysis (Fig. 15) and EIA (Fig. 16), in which [^{14}C]-AA (for HPLC assay) or unlabeled AA (for ELISA assay) was used as a substrate for the cells over-expressing the engineered protein. Fig. 15 is a bar graph showing the determination of the tri-catalytic activities of the recombinant proteins directly converting AA to PGE_2 using an HPLC method for HEK293 cells. The cells were transfected with the recombinant cDNAs of COX-2–10AA–mPGES (A), COX-2 and mPGES (B), COX-2 only (C), mPGES only (D), or pcDNA 3.1 vector only (E). The untransfected cells were also used as a control (F). The addition of a COX-2 inhibitor (NS-398, 10 μM) to the cells transfected with COX-2-10aa-mPGES cDNA is also shown (G). The cells were washed three times, then 25 μl were suspended in 2.5mM GSH and then incubated with [^{14}C]-AA in a total volume of 225 μl. After two min, the reaction was terminated by the addition of 2mM $SnCl_2$ and 0.1% TFA (200 μl each) and then centrifuged at 6,000 rpm for 5 min. The supernatant was separated by HPLC on a C18 column (4.5 x 250 mm) using buffer A with a gradient of 35-100% acetonitrile. The [^{14}C]-AA metabolites were determined by a liquid scintillation analyzer built in the HPLC system. The retention time of [^{14}C]-PGE_2 and [^{14}C]-AA were calibrated by standards under the same conditions. The time represented above in the HPLC data was measured in minutes and CPM stands for counts per minute. The tri-catalytic activities in the engineered single protein were identical to the reactions mediated by two enzyme proteins of COX-2 and mPGES co-expressed in the cells (Fig. 14-15). Thus, the COX-2–10aa–mPGES indeed exhibited tri-catalytic activities in the direct conversion of AA to PGE_2. This biological activity was further confirmed by an inhibition assay using a COX-2 inhibitor, NS-398 (Fig. 15G). Additional controls include the COX-2 enzyme alone (Fig. 15C) which primarily produced $PGF_{2\alpha}$ as well as a small amount of PGE_2. The mPGES protein was also tested and failed to produce PGE_2 using AA as a substrate (Fig 15D).

Referring now to Fig. 16, it was determined using Enzyme Immunoassay (EIA) that the tri-catalytic activities of the recombinant proteins directly converting AA to PGE_2. After the enzyme reactions described in Fig. 15 for the reaction mixtures prepared from the cells transfected with the plasmid of the COX-2–10aa–mPGES (Fig. 16, Bar 1), the mixture of the plasmids of the COX-2 and mPGES (Fig. 16, Bar 2), or the pcDNA3.1 vector alone (Fig. 16, Bar 3) were diluted 100 times with PBS containing 0.1% BSA, and then used for quantitative determination of PGE_2 using an EIA kit following the instructions of the manufacturer (Cayman, Ann Arbor, MI).

These results are evidence of the conversion of AA into PGE_2 by the single protein COX-2–10aa–mPGES using unlabeled AA as a substrate for the cells over-expressing the engineered protein is demonstrated.

Example 8.

Directly Converting dihomo-γ-linolenic acid (DGLA) into PGE_1, by the Engineered COX-2-10aa-mPGES with Triple Catalytic Activities

Another endogenous polyunsaturated fatty acid, DGLA (20:3 *n*–6), is also a substrate for COX-2–10aa–mPGES and yields PGE_1, which has been identified as an important endogenous and exogenous molecule with anti-inflammatory properties. PGE_1 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 PGE_1 by the single protein of COX-2–10aa–mPGES was obtained by HPLC analysis. The produced PGE_1 was further identified by an above-described standard method.

Referring to Figs. 17A-B, determination of the tri-catalytic activities of the recombinant proteins directly converting DGLA to PGE_1 using an HPLC method for HEK293 cells is shown. The cells were transfected with the recombinant cDNA COX-2–10AA–mPGES (Fig. 17A), or the recombinant cDNAs of COX-2 and mPGES (Fig. 17B). The methods used for assay and HPLC analysis were as described above with respect to Fig. 15. The time represented in the HPLC data was measured in minutes and CPM stands for counts per minute. Thus, the COX-2–10aa–mPGES also exhibited tri-catalytic activities in the direct conversion of DGLA to PGE_1. PGE_1 has similar biological functions to PGI_2 and is expected to be similarly useful therapeutically, as discussed above with respect to the representative COX-linker-PGIS engineered protein.

While the present invention has been described in reference to illustrative embodiments, this description is not intended to be construed in a limiting sense. Various modifications and combinations of the illustrative embodiments, as well as other embodiments of the invention, will be apparent to persons skilled in the art upon reference to the description. For example, in the foregoing examples, certain 10aa and 22aa linker peptides are employed. The results obtained with those linker sequences are considered representative of other peptides with different sequences and/or intermediate sequence lengths between 10aa and 22aa that may also serve as satisfactory linker sequences. Such other satisfactory sequences, in a similar hybrid protein, must be able to span the cell membrane and permit the COX and PGIS or PGES to assume membrane topographies similar to their native counterpart enzymes, to fold correctly, and to demonstrate enzymatic activity similar to that of the native COX and PGIS or PGES. Further, this disclosure provides evidence that the COX enzymes can be linked with other downstream eicosanoid-synthesizing (ES) enzymes, thereby generating other linked proteins, to specifically regulate the biosynthesis of eicosanoids, which play diverse and potent biological functions in human. Thus,

PGIS and PGES are considered to be representative of other eicosanoid-synthesizing enzymes that may be used to construct similarly successful hybrid proteins. It is therefore intended that the appended claims encompass any such modification or embodiments. The disclosures of all patents, patent applications and publications cited herein are hereby incorporated herein by reference, to the extent that they provide exemplary, procedural or other details supplementary to those set forth herein.

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CLAIMS

What is claimed is:

1. An isolated hybrid protein molecule comprising a cyclooxygenase (COX) amino acid sequence and a prostaglandin E_2 synthase (PGES) amino acid sequence, with a linker sequence disposed therebetween and directly connecting said COX enzyme sequence to said ES enzyme sequence.

2. The hybrid protein molecule of claim 1 wherein said linker sequence is capable of functioning as a transmembrane linker in a cell such that the folding ability and functions of the COX and the PGES are substantially unaltered compared to the folding ability and function of the respective native enzymes.

3. The hybrid protein molecule of claim 2 wherein said linker sequence is about 10 to 22 amino acids long.

4. The hybrid protein molecule of claim 1 wherein said linker sequence is His-Ala-Ile-Met-Gly-Val-Ala-Phe-Thr-Trp (SEQ ID NO. 1).

5. The hybrid protein molecule of claim 1 wherein said linker sequence is His-Ala-Ile-Met-Gly-Val-Ala-Phe-Thr-Trp-Val-Met-Ala-Leu-Ala-Cys-Ala-Ala-Pro-Pro-Leu-Val (SEQ ID NO. 2).

6. The hybrid protein molecule of claim 1 wherein said linker sequence is residues 1-10, 1-11, 1-12, 1-13, 1-14, 1-15, 1-16, 1-17, 1-18, 1-19, 1-20 or 1-21 of SEQ ID NO. 2.

7. The hybrid protein of claim 6 having the general structure COX-2–linker–mPGES.

8. The hybrid protein of claim 1, wherein said linker peptide provides approximately 10 Å separation between the catalytic sites of said COX and PGES enzymes, said connected enzymes being capable of substantially normal folding and enzymatic activity compared to the native folding and enzymatic activity of the native COX and PGES enzymes.

9. The hybrid protein of claim 1, wherein said hybrid protein is chemically synthesized.

10. The hybrid protein of claim 1, wherein said hybrid protein is recombinantly produced.

11. A pharmaceutical composition comprising:
the hybrid protein of any of claims 1-10; and
a pharmaceutically acceptable carrier.

12. An isolated DNA sequence encoding:
a cyclooxygenase (COX);
a transmembrane linker peptide; and
a prostaglandin E_2 synthase (PGES).

13. The isolated DNA sequence of claim 12, wherein the cyclooxygenase is a cyclooxygenase isoform-1 (COX-1).

14. The isolated DNA sequence of claim 12, wherein the cyclooxygenase is a cyclooxygenase isoform-2 (COX-2).

15. The isolated DNA sequence of claim 12, having the general structure COX-linker-mPGES.

16. The isolated DNA sequence of claim 12 wherein the transmembrane linker peptide has the amino acid sequence His-Ala-Ile-Met-Gly-Val-Ala-Phe-Thr-Trp (SEQ ID NO. 1).

17. The isolated DNA sequence of claim 12, wherein the transmembrane linker peptide has the amino acid sequence His-Ala-Ile-Met-Gly-Val-Ala-Phe-Thr-Trp-Val-Met-Ala-Leu-Ala-Cys-Ala-Ala-Pro-Pro-Leu-Val (SEQ ID NO. 2).

18. The isolated DNA sequence of claim 12, wherein the transmembrane linker peptide is 11 to 21 amino acid residues in length and corresponds to residues 1-11, 1-12, 1-13, 1-14, 1-15, 1-16, 1-17, 1-18, 1-19, 1-20 or 1-21 of SEQ ID NO. 2.

19. A virus vector comprising the DNA sequence according to any of claims 12-18.

20. The vector of claim 19 wherein the virus is adeno-associated virus.

21. A host cell containing an expressible DNA sequence according to any of claims 12-18.

22. A process for producing a hybrid protein comprising a cyclooxygenase (COX), a transmembrane linker, and a prostaglandin E_2 synthase (PGES), the process comprising:

culturing the host cell of claim 21 under conditions suitable for expression of the DNA encoding said hybrid protein molecule, and

recovering a biologically active hybrid protein molecule comprising a cyclooxygenase (COX), a transmembrane linker, and a prostaglandin E_2 synthase (PGES).

23. The process of claim 22, wherein said recovered hybrid protein comprises enzymatically active cyclooxygenase, transmembrane linker, and enzymatically active prostaglandin E_2 synthase (PGES).

24. The process of claim 22 comprising transfecting said host cell with a vector comprising said DNA sequence prior to said culturing.

25. A method of producing PGE_1 comprising converting dihomo-γ-linolenic acid to PGE_1 with the hybrid molecule of any of claims 1-10.

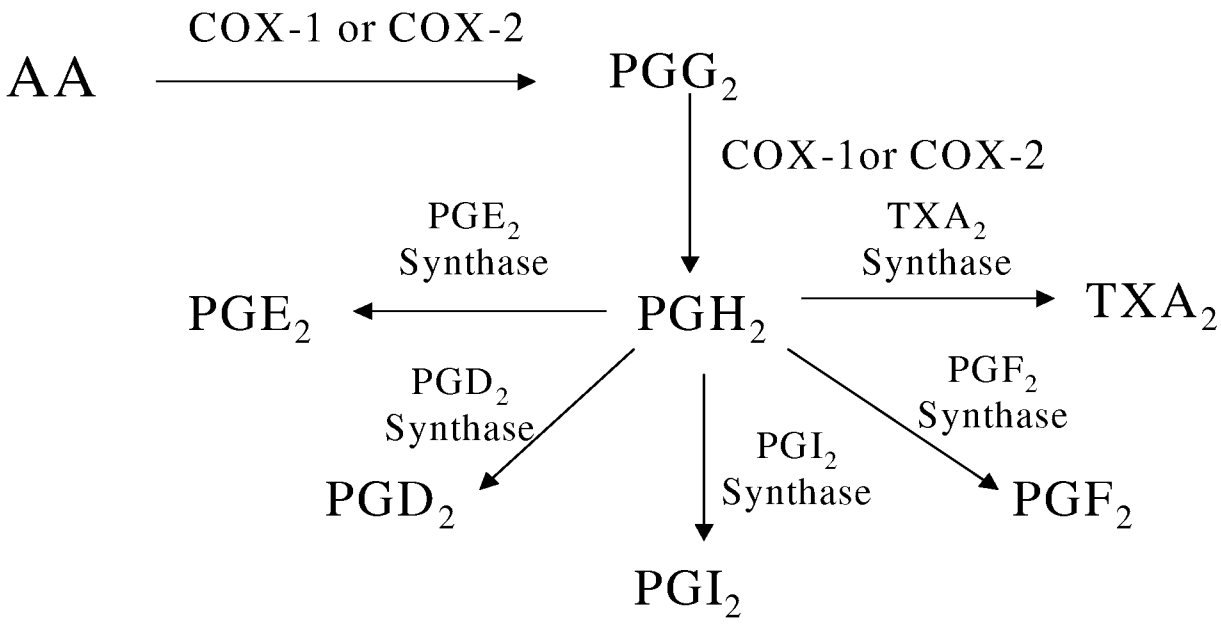

AA
COX-1 or COX-2
PGG2
COX-1or COX-2
PGE2 Synthase
TXA2 Synthase
PGE2
PGH2
TXA2
PGD2 Synthase
PGF2 Synthase
PGI2 Synthase
PGD2
PGF2
PGI2

FIG. 1

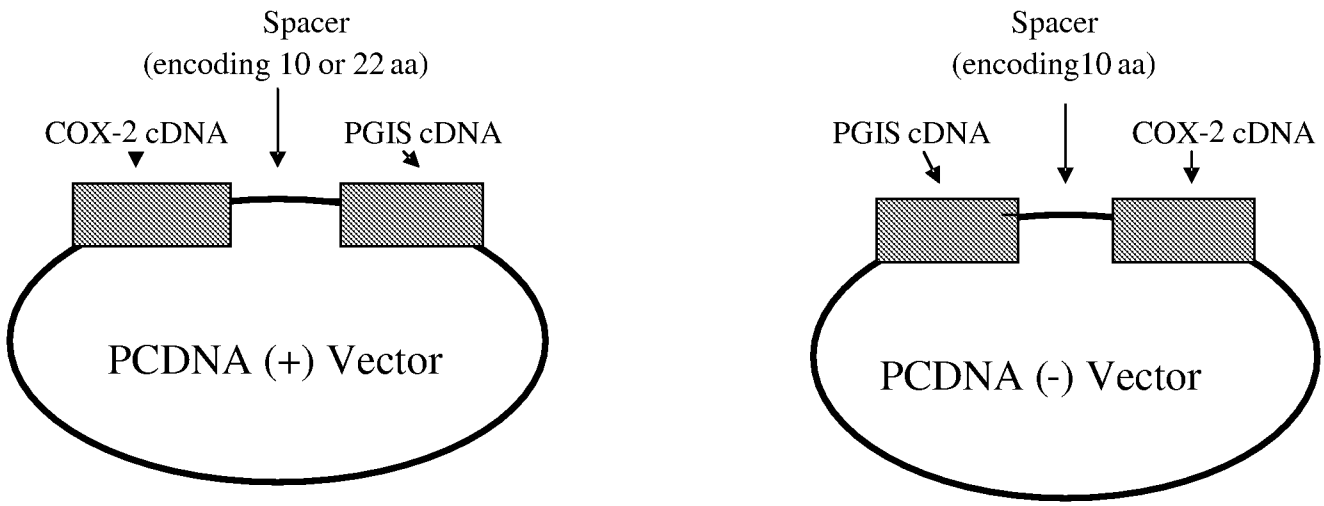

Spacer
(encoding 10 or 22 aa)
COX-2 cDNA
PGIS cDNA
PCDNA (+) Vector
Spacer
(encoding10 aa)
PGIS cDNA
COX-2 cDNA
PCDNA (-) Vector

FIG. 2

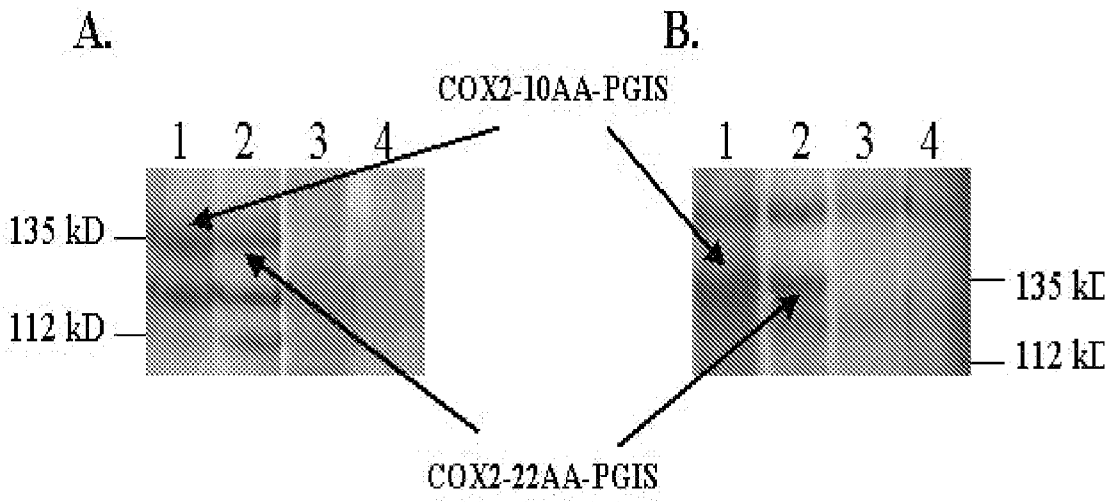

A.
B.
COX2-10AA-PGIS
1 2 3 4
1 2 3 4
135 kD
112 kD
135 kD
112 kD
COX2-22AA-PGIS

FIG. 3

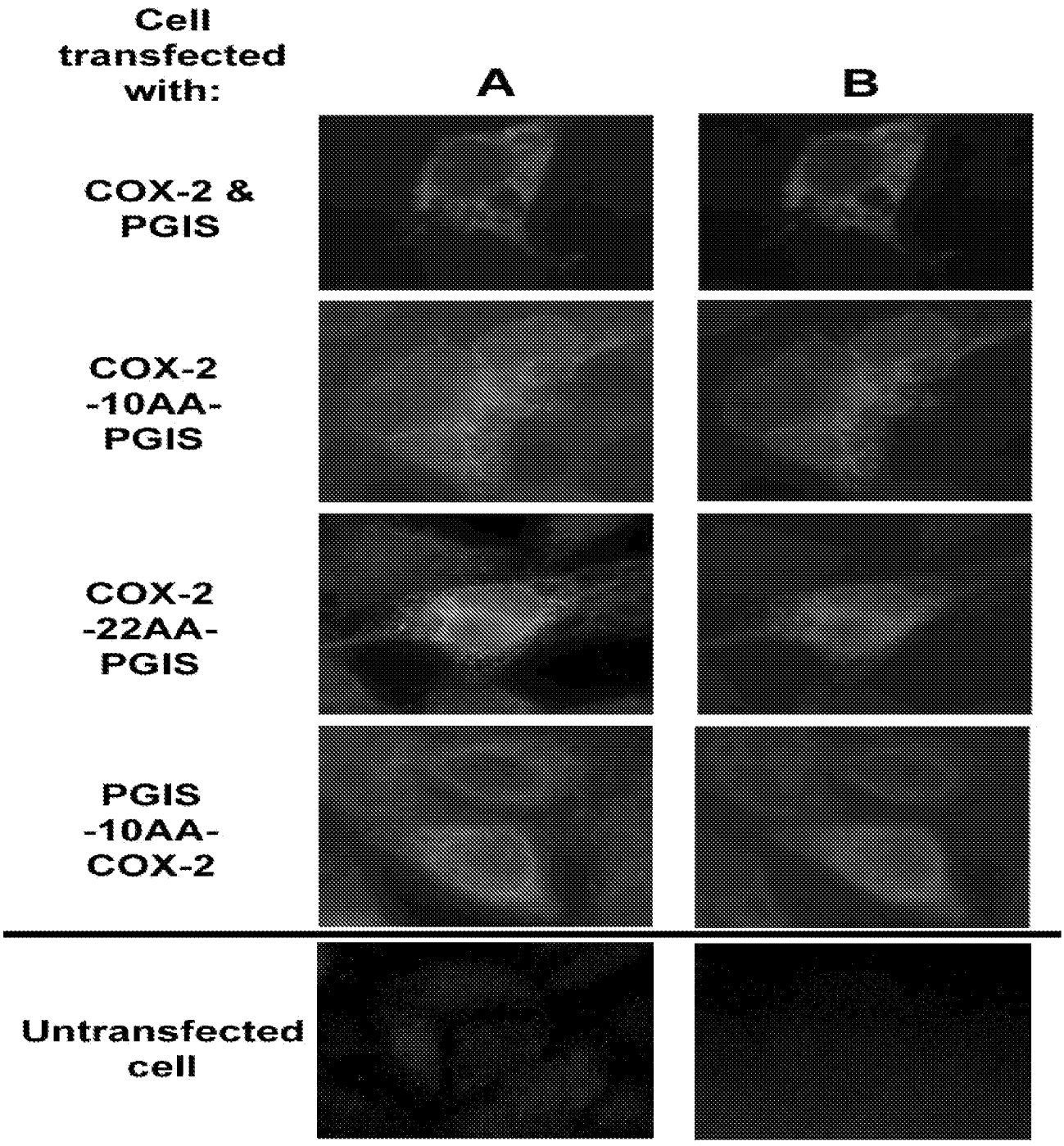

Cell transfected with:
A
B
COX-2 & PGIS
COX-2 -10AA- PGIS
COX-2 -22AA- PGIS
PGIS -10AA- COX-2
Untransfected cell

FIG. 4

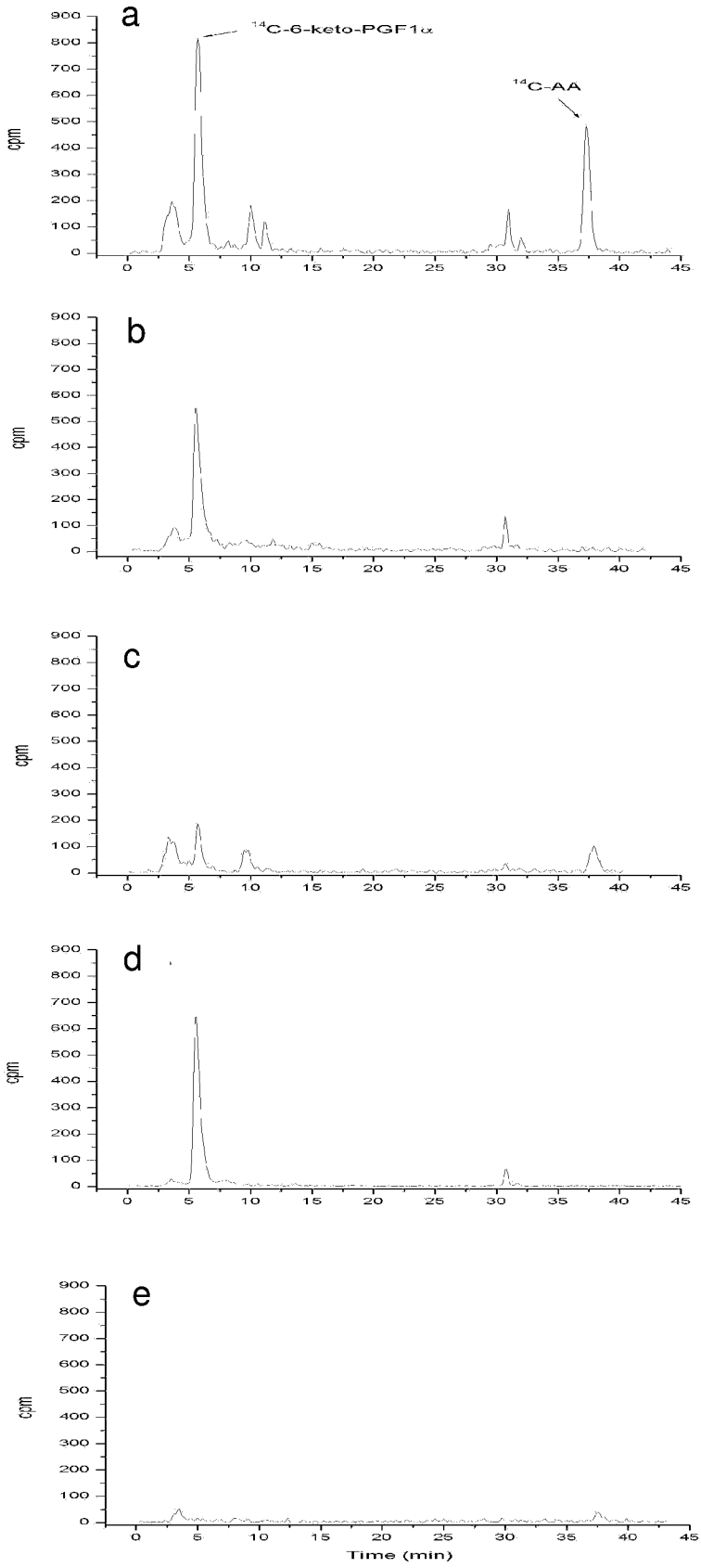


FIG. 5A

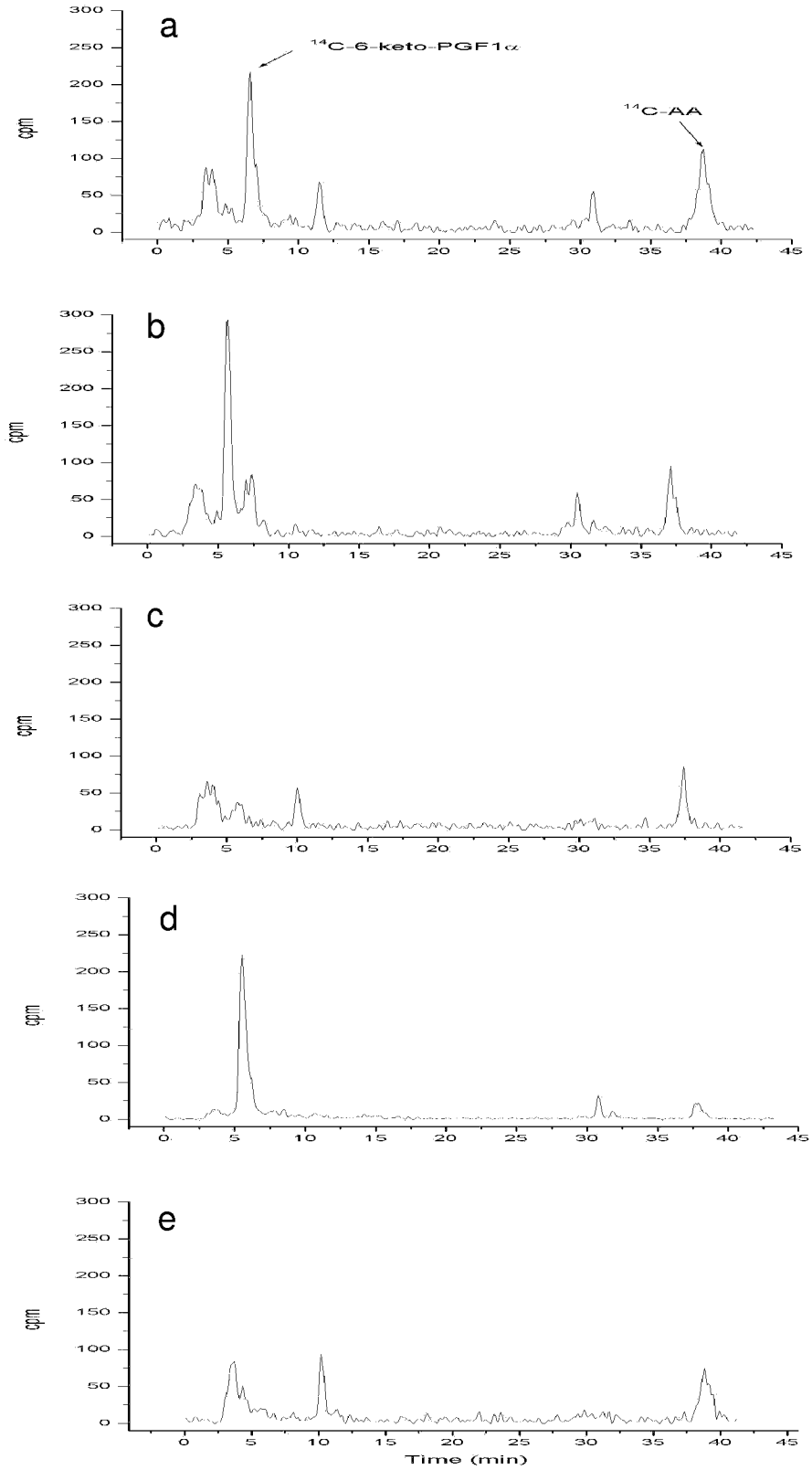


FIG. 5B

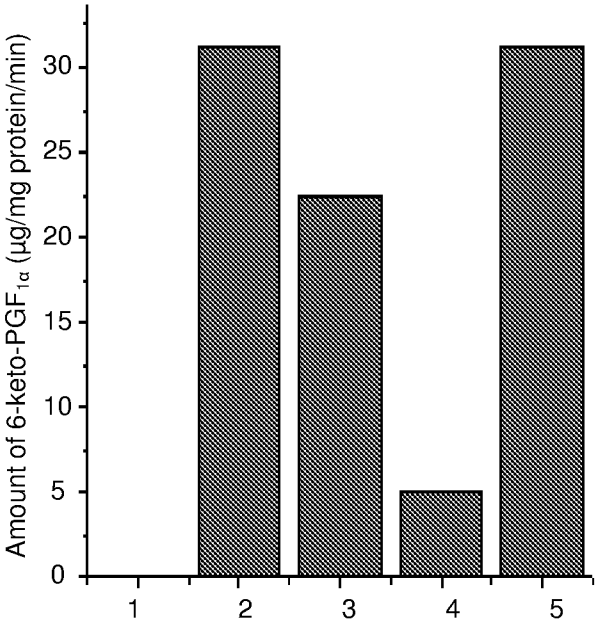

Amount of 6-keto-PGF1α (µg/mg protein/min)
0
5
10
15
20
25
30
1
2
3
4
5

FIG. 6A

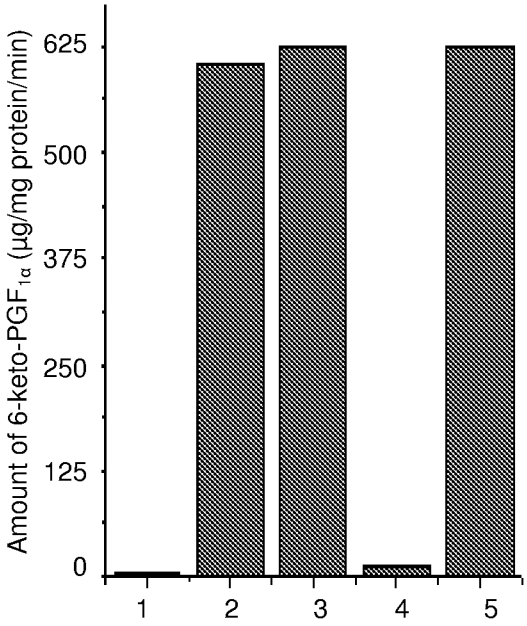

Amount of 6-keto-PGF1α (µg/mg protein/min)
0
125
250
375
500
625
1
2
3
4
5

FIG. 6B

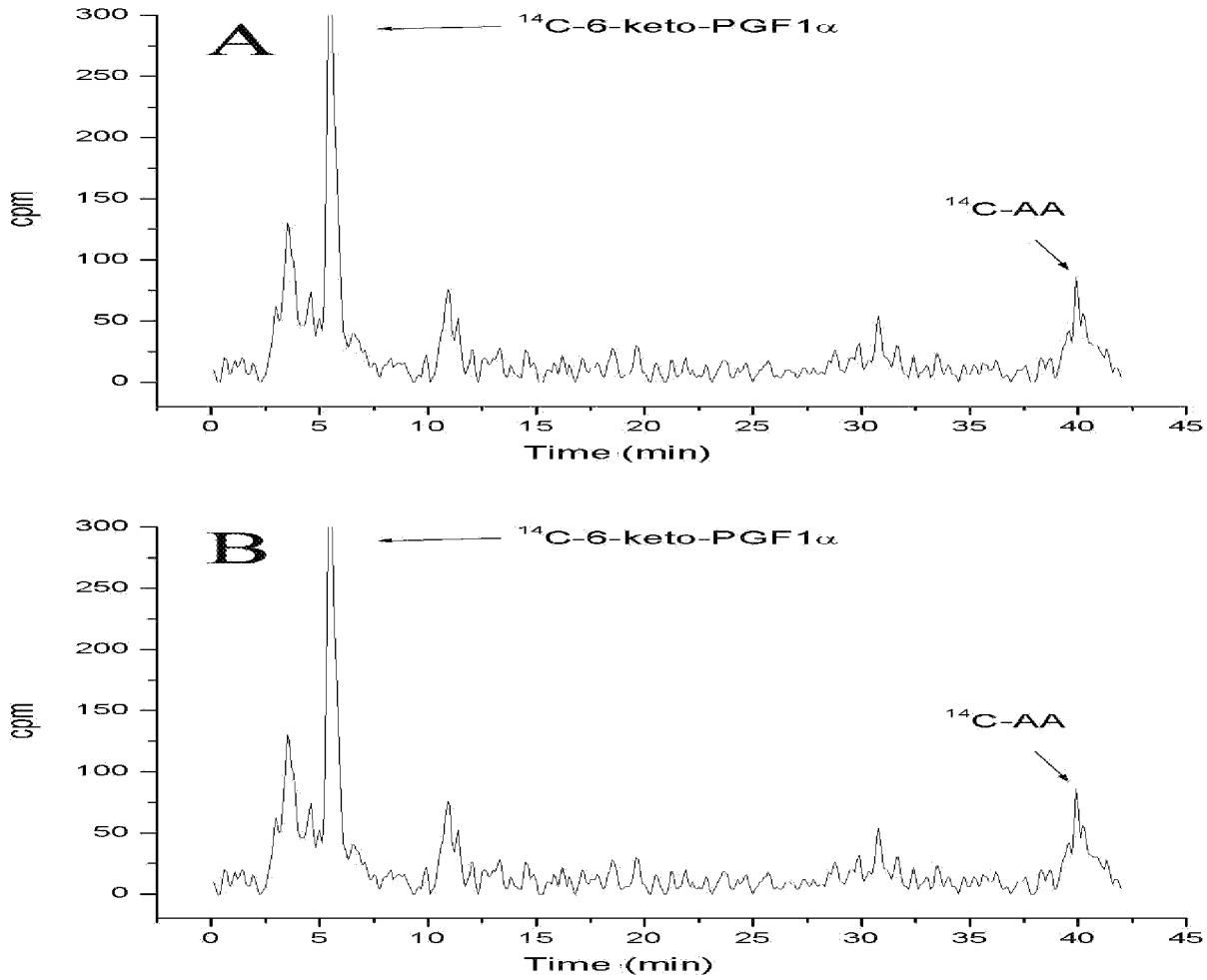

A
^{14}C-6-keto-PGF1α
^{14}C-AA
cpm
Time (min)
B
^{14}C-6-keto-PGF1α
^{14}C-AA
cpm
Time (min)

FIG. 7

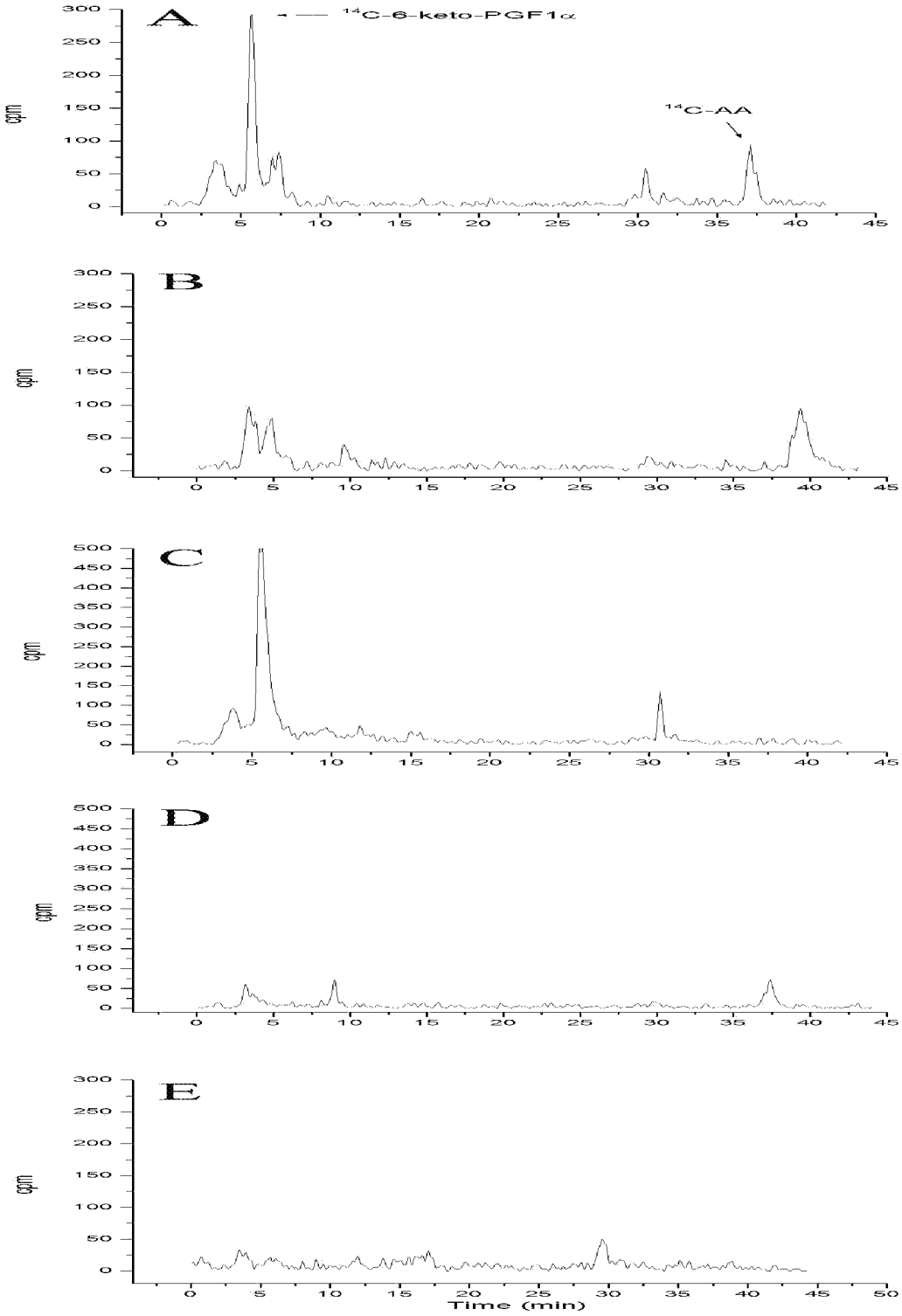


FIG. 8

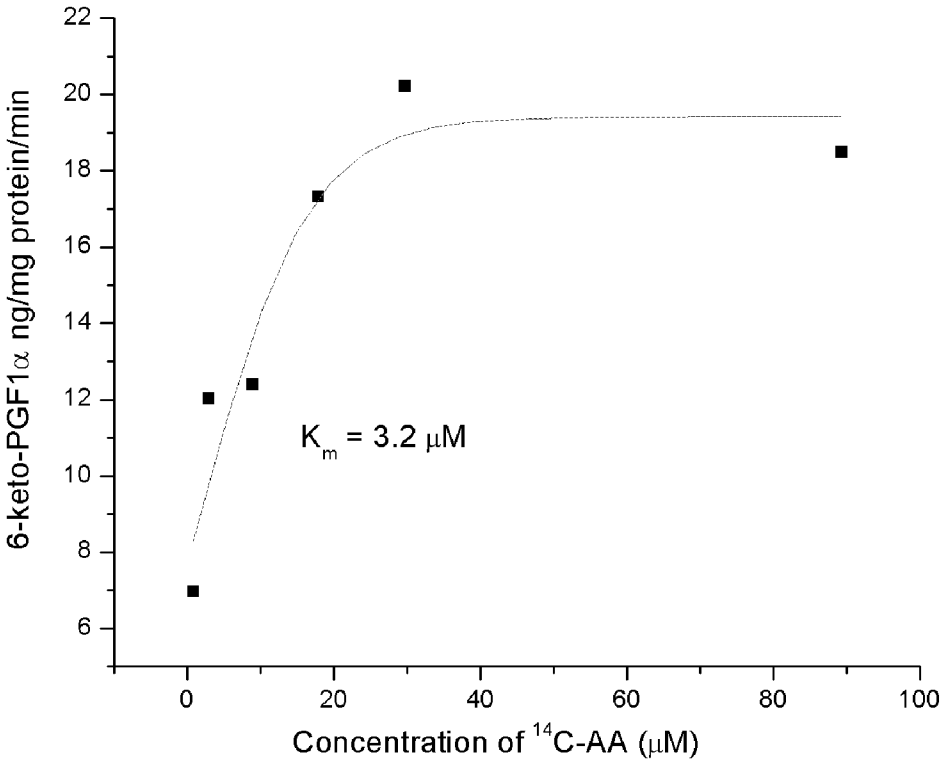

6-keto-PGF1α ng/mg protein/min
22
20
18
16
14
12
10
8
6
0
20
40
60
80
100
Concentration of ^{14}C-AA (μM)
K_m = 3.2 μM

FIG. 9

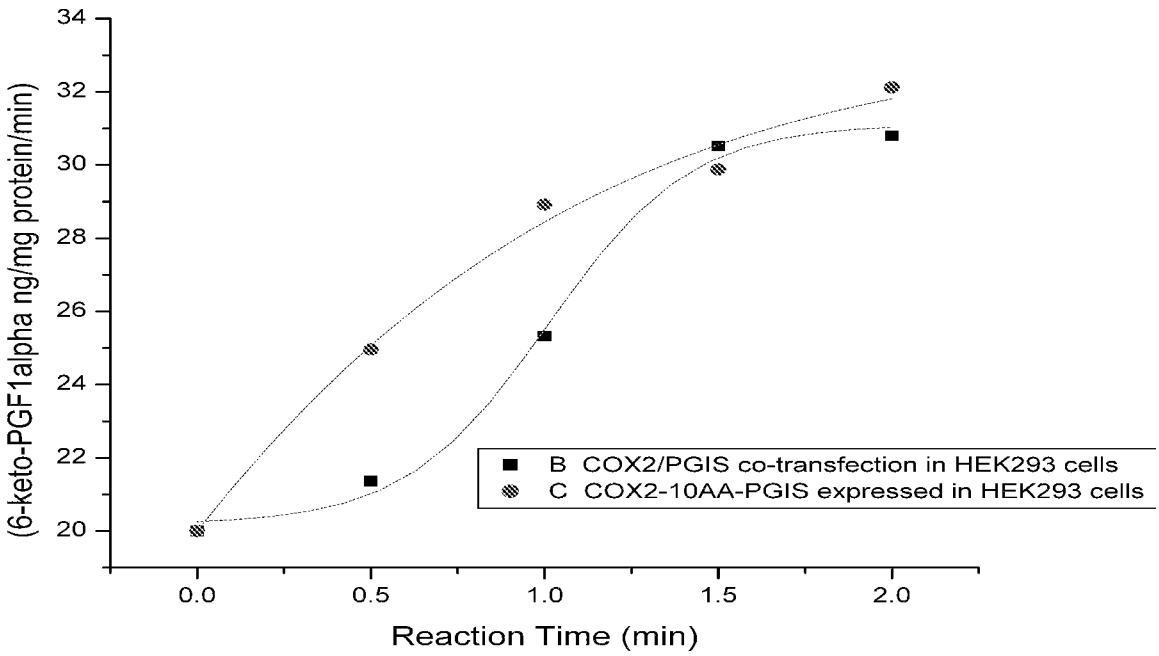
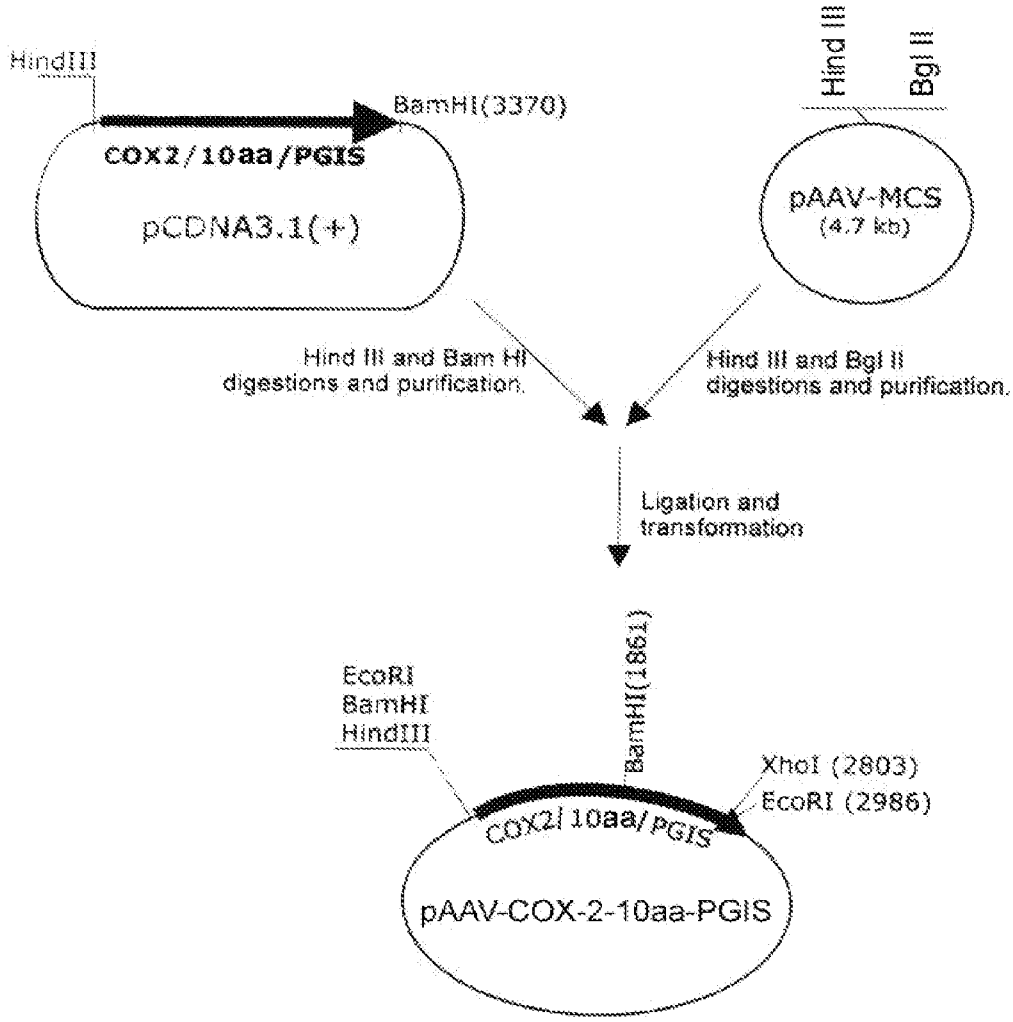

(6-keto-PGF1alpha ng/mg protein/min)
34
32
30
28
26
24
22
20
0.0
0.5
1.0
1.5
2.0
Reaction Time (min)
B COX2/PGIS co-transfection in HEK293 cells
C COX2-10AA-PGIS expressed in HEK293 cells

FIG. 10

A.



B.

Agarose gel separations for the digested plasmid containing the COX-2-10aa-PGIS cDNA →

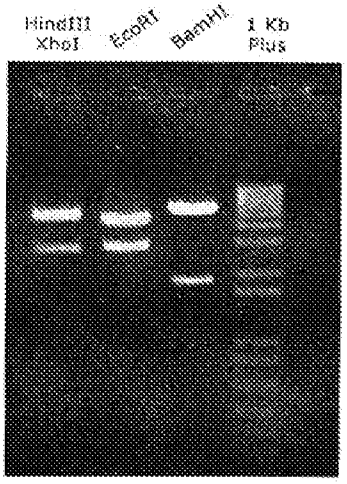


FIG. 11

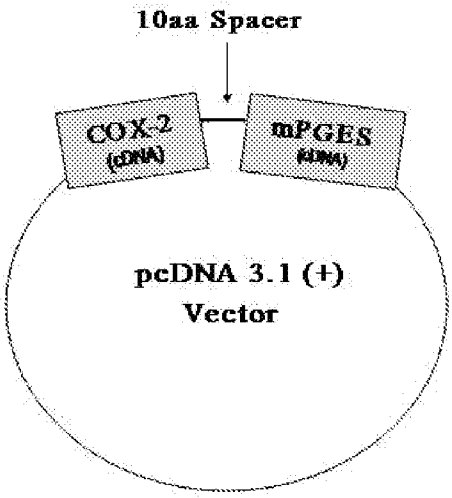

10aa Spacer
COX-2
(cDNA)
mPGES
pcDNA 3.1 (+)
Vector

FIG. 12

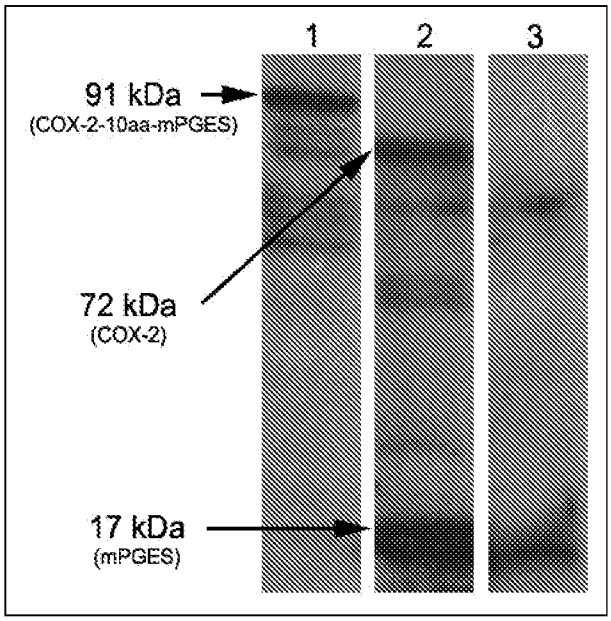

1
2
3
91 kDa
(COX-2-10aa-mPGES)
72 kDa
(COX-2)
17 kDa
(mPGES)

FIG. 13

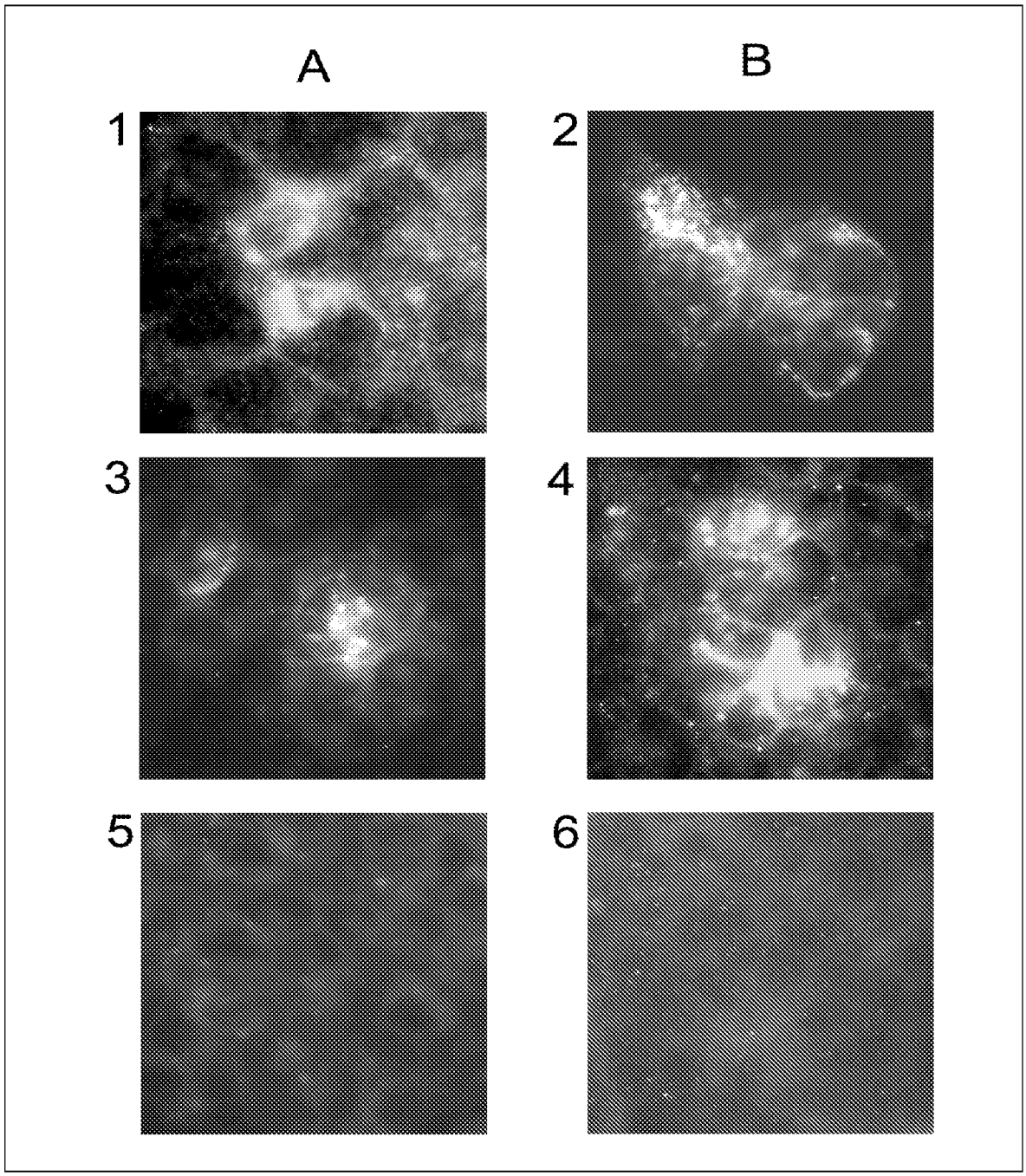


FIG. 14

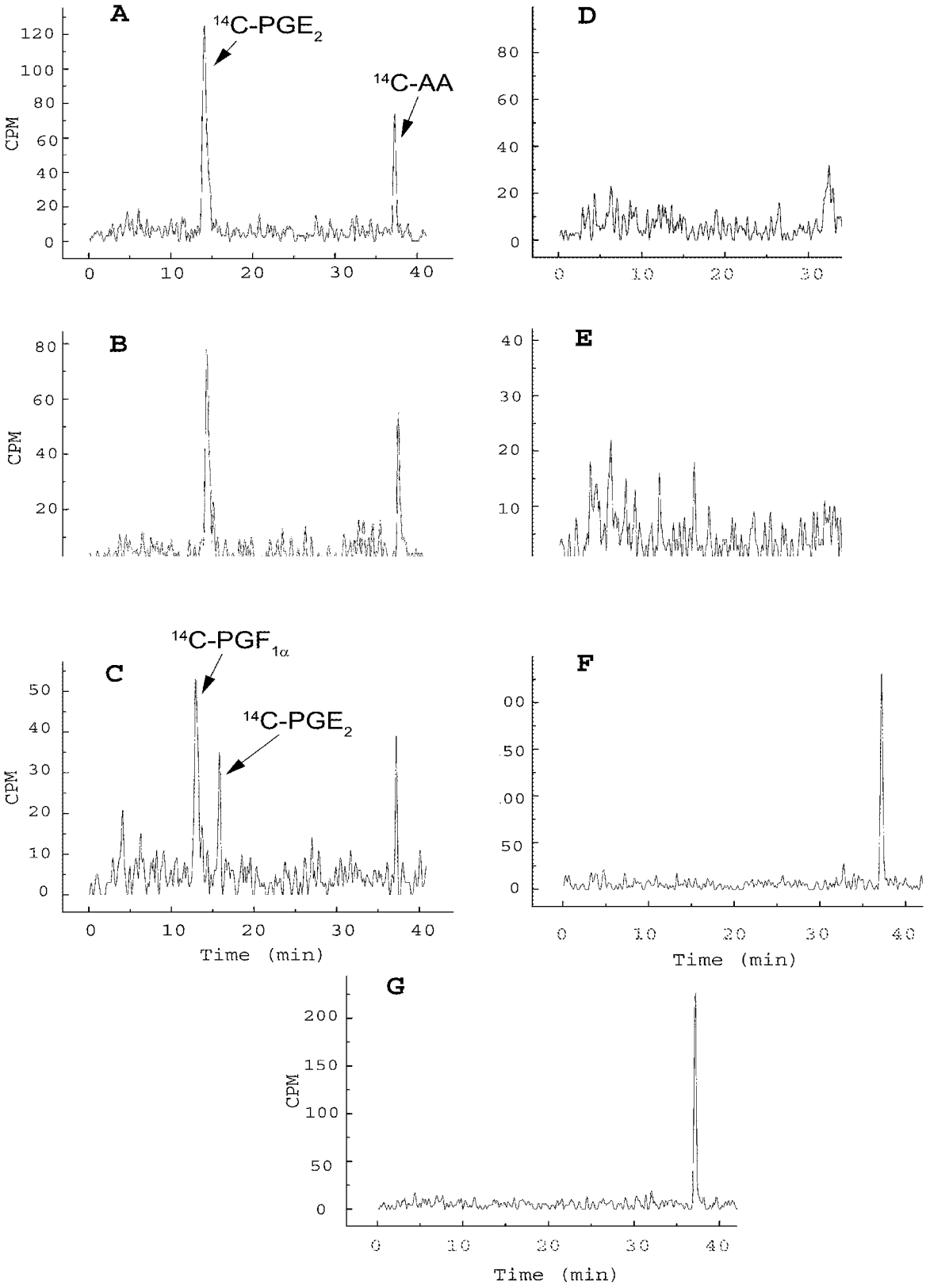

A
^{14}C-PGE$_2$
^{14}C-AA
CPM
B
C
^{14}C-PGF$_{1\alpha}$
^{14}C-PGE$_2$
Time (min)
D
E
F
Time (min)
G
Time (min)

FIG. 15

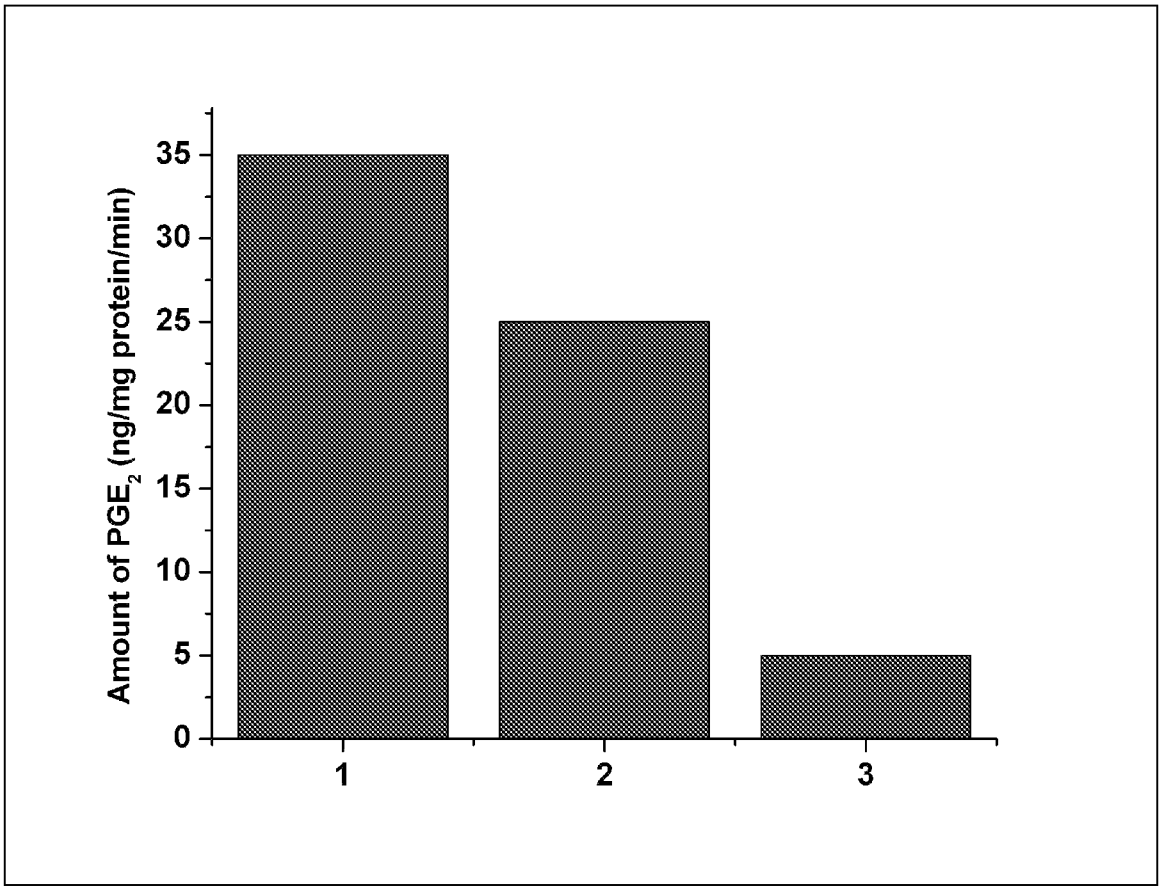


FIG. 16

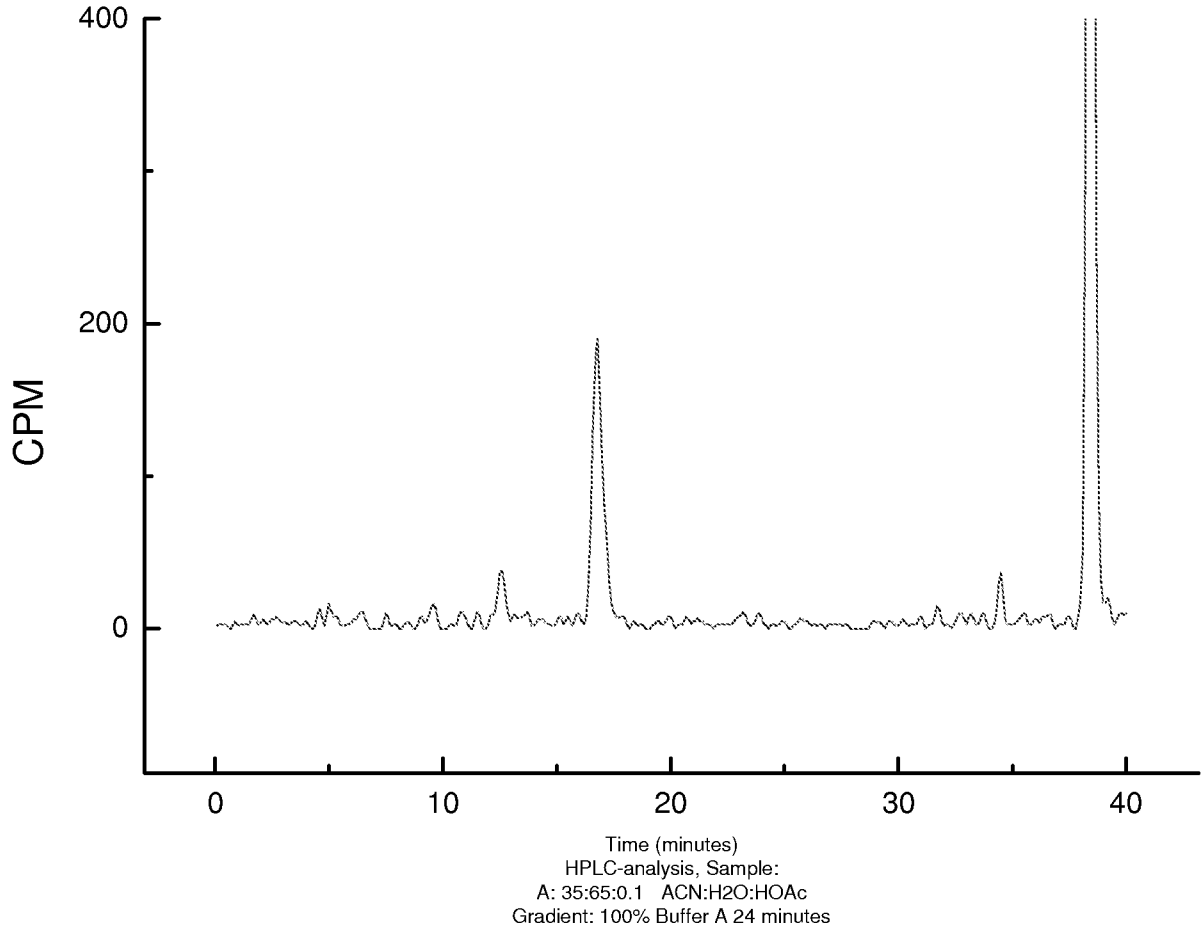

400
200
0
CPM
0
10
20
30
40
Time (minutes)
HPLC-analysis, Sample:
A: 35:65:0.1 ACN:H2O:HOAc
Gradient: 100% Buffer A 24 minutes

FIG. 17A

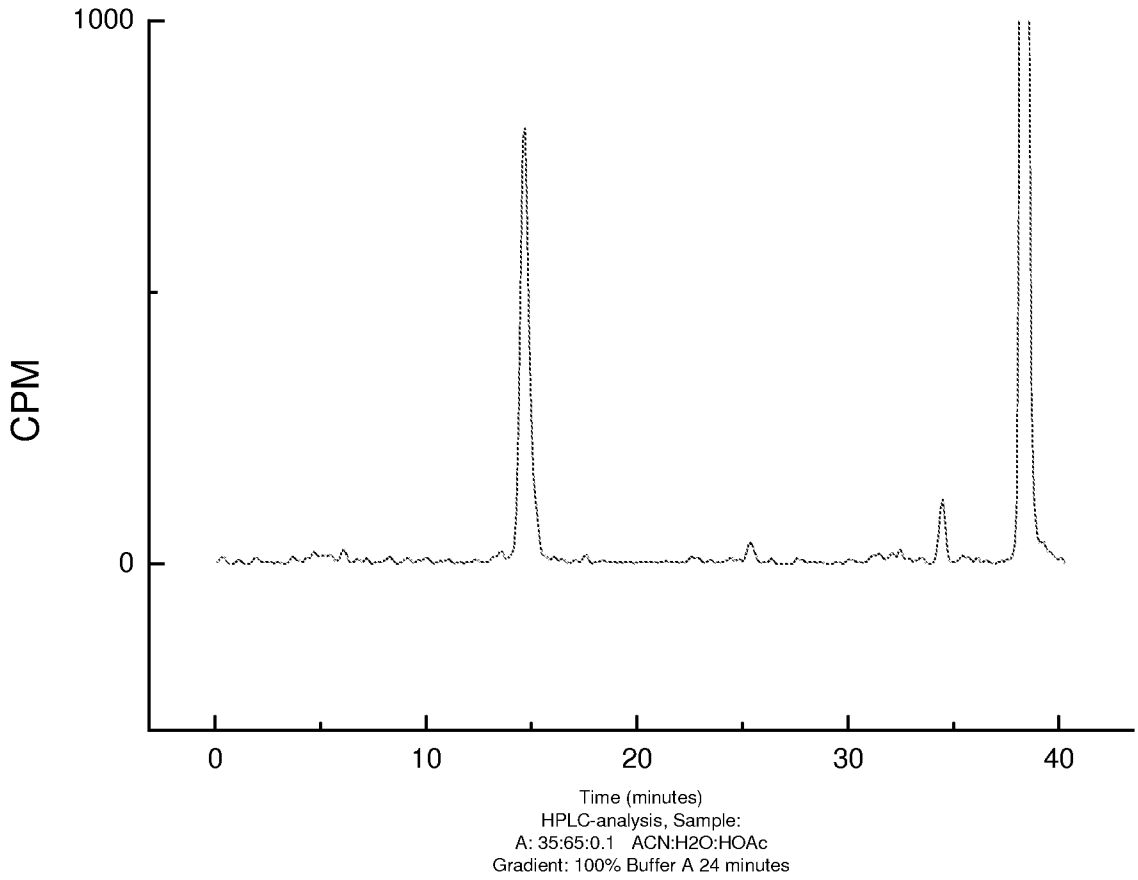

1000
0
CPM
0
10
20
30
40
Time (minutes)
HPLC-analysis, Sample:
A: 35:65:0.1 ACN:H2O:HOAc
Gradient: 100% Buffer A 24 minutes

FIG. 17B