Identification of the Two-Phase Mechanism of Arachidonic Acid Regulating Inflammatory Prostaglandin E2 Biosynthesis by Targeting COX-2 and mPGES-1

Hironari Akasaka ^{a,1}, and Ke-He Ruan ^{a,1,*}

^a Department of Pharmacological and Pharmaceutical Sciences, University of Houston College of Pharmacy, Houston, TX 77204-5037, USA

¹ This author takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

* **Corresponding Author:** Ke-He Ruan, M.D., Ph.D. University of Houston, Department of Pharmacological and Pharmaceutical Sciences. 552 Science and Research Building 2, 4800 Calhoun Road, Houston, Texas 77204-5037. Phone 713-743-1771. Fax 713-743-1229. Email: khruan@uh.edu

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- **Abbreviations**

10 amino acid residues, 10aa; arachidonic acid, AA; coronary heart disease, CHD; cyclooxygenase-1 and -2, COX-1 and -2, respectively; cytosolic PGES, cPGES; epoxyeicosatrienoic acid, EET; high performance liquid chromatography, HPLC; human embryonic kidney, HEK; microsomal PGE synthase-1 and -2, mPGES-1 and -2, respectively; nonsteroidal anti-inflammatory drugs, NSAIDs; polyunsaturated fatty acid, PUFA; prostacyclin or prostaglandin I₂, PGI₂; prostaglandin E₂, PGE₂; prostaglandin H₂, PGH₂; Protein Data Bank, PDB; Single-chain Enzyme Complex, SCEC; thromboxane A₂, TXA₂.

Abstract

Through linking inducible cyclooxygenase (COX)-2 with microsomal prostaglandin E2 (PGE₂) synthase-1 (mPGES-1), a Single-Chain Enzyme Complex (SCEC, COX-2-10aa-mPGES-1) was engineered to mimic a specific inflammatory PGE₂ biosynthesis from omega-6 fatty acid, arachidonic acid (AA), by eliminating involvements of non-inducible COX-1 and other PGE₂ synthases. Using the SCEC, we characterized coupling reactions between COX-2 and mPGES-1 at 1:1 ratio of inflammatory PGE₂ production. AA demonstrated two phase activities to regulate inflammatory PGE₂ production. In the first phase (< 2 μM), AA was a COX-2 substrate and converted to increasing production of PGE₂. In the second phase with a further increased AA level (2-10 μM), AA bound to mPGES-1 and inhibited the PGE₂ production. The SCEC study was identical to the co-expression of COX-2 and mPGES-1. This was further confirmed by using mPGES-1 and PGH₂ as a direct enzyme target and substrate, respectively. Furthermore, the carboxylic acid group of AA binding to R67 and R70 of mPGES-1 was identified by X-ray structure-based docking and mutagenesis. mPGES-1 mutants, R70A, R70K, R67A and R67K, lost 40-100% binding to [¹⁴C]-AA. To conclude, a cellular model, in which AA is involved in self-controlling initial initiating and later resolving inflammation by its two phase activities, was discussed.

1. Introduction

Three PGE₂ synthases, microsomal prostaglandin E₂ (PGE₂) synthase (mPGES)-1, mPGES-2 and cytosolic PGE₂ synthase (cPGES), have been cloned¹⁻⁴. mPGES-1 is the major enzyme involved in producing inflammatory PGE₂ through instantly accepting the inducible cyclooxygenase (COX)-2-produced PGH₂ as a substrate. In addition, we have recently identified that COX-2 and mPGES-1 have a complex-like configuration anchored on the cellular ER membrane to effectively catalyze arachidonic acid (AA) to PGE₂ causing inflammation⁵. Thus, it is reasonable to posit that giving more omega-6 polyunsaturated fatty acid (PUFA), such as linoleic acid (precursor of AA), would advance tissue inflammation by acting on the COX-2/mPGES-1 system to produce more inflammatory PGE₂. However, recent animal and clinical trial studies have indicated that increasing daily consumption of omega-6 PUFA to at least 5-10% of total dietary intake likely decreases the risk of coronary heart disease (CHD)⁶. The molecular mechanisms behind the results are poorly understood.

Considering the major omega-6 PUFA is linoleic acid (consisting of 80-90% of the PUFA diet), which is converted into AA *in vivo*, investigation of AA metabolites in cells would further uncover the molecular mechanisms of the PUFA effects on reducing CHD. Three upstream enzymes, COX, lipoxygenase and cytochrome P450, are known to use AA as a substrate⁷. In a COX pathway, AA is metabolized into prostanoids, including prostacyclin (PGI₂), thromboxane A₂ (TXA₂), PGE₂ and others, to maintain healthy physiological functions⁸. However, in inflammatory conditions (such as vascular inflammation, arthritis and some cancers), AA is metabolized into a biased PG production toward PGE₂ to mediate the pathological processes, while reducing the amount of other prostanoids^{9,10}. In lipoxygenase and cytochrome P450 pathways, AA is metabolized into leukotrienes and epoxyeicosatrienoic acids

(EETs), having an impact on mediating asthma and vascular protection, respectively ¹¹. However, the COX pathway seems to be a dominated AA metabolism, observed by the inhibition of COXs by non-steroid anti-inflammatory drugs (NSAIDs) that leads to a dramatic increase in cellular AA concentration ¹². Thus, characterizing the molecular and cellular activities of AA in detail shall be useful to further understand the cellular effects of omega-6 PUFAs on inflammatory diseases.

In the past, AA activity was studied under the co-expression condition of inducible COX-2 and mPGES-1 ^{13,14}. The uneven co-expression of COX-2 and mPGES-1, and the involvement of the other two PGE₂ synthases, cPGES and mPGES-2, became the limitation for quantifying how exact AA binding behaves from one molecule of COX-2 to one mPGES-1, and also the limitation for studying whether AA regulates the PGE₂ production in addition to being used as a substrate for COX-2. In this study, using our engineered Single-chain Enzyme Complex (SCEC), COX-2-10aa-mPGES-1, which possesses three catalytic domains in a single polypeptide chain, we were able to limit the involvement of non-inducible PGE₂ synthases, cPGES and mPGES-2, to characterize AA metabolites during cellular inflammation. It led us to find the concentration-dependent of two-phase mechanism of AA, regulating cellular inflammatory PGE₂ biosynthesis as a COX-2 substrate and an mPGES-1 inhibitor at molecular and structural levels. The study has provided novel mechanisms to explain that a higher intake of omega-6 PUFAs could be even more beneficial to reduce vascular inflammation and CHD, which is highly interesting to public and medical communities in general.

2. Material and methods

2.1. Materials

HEK293 cell line was purchased from ATCC (Manassas, VA). Dulbecco's modified Eagle's medium, Geneticin, and Antibiotic-Antimycotic (100X) for culturing the cell lines and lipofectamine 2000 for transfecting cDNAs were purchased from Invitrogen (Carlsbad, CA). Fetal Bovine Serum (FBS) was from Life Technologies (Grand Island, NY). [^{14}C]-AA was purchased from PerkinElmer (Waltham, MA). [^3H]-PGH₂ and the primary antibodies for human COX-2 and mPGES-1 were purchased from Cayman Chemical Company (Ann Arbor, MI). The primary antibody of β -actin was purchased from Cell Signaling Technology (Danvers, MA). Primers for mPGES-1 mutants were purchased from Sigma-Aldrich, Inc (Staint Louis, MO). pcDNATM3.1 (+) was purchased from Invitrogen Corporation (Carlsbad, CA).

2.2. Establishing HEK293 cell lines expressing recombinant enzymes

The cDNA of the SCEC was previously reported from our group¹⁵. Briefly, HEK293 cells were transfected with the purified cDNA of the recombinant synthases (co-transfection with COX-2 and mPGES-1, transfection with COX-2-10aa-mPGES-1, mPGES-1, or mPGES-1 mutants) by the Lipofectamine 2000 method, following the manufacturer's instructions (Invitrogen). Approximately 48 hours after transfection, HEK-COX-2/mPGES-1 cells were harvested for further enzyme assays. For the stable expression, the cells were incubated with culture medium (10 % FBS, 1% Antibiotic-Antimycotic (100X) in DMEM containing geneticin (G418) 12 $\mu\text{L}/\text{mL}$).

2.3. Enzyme activity determination of HEK-COX-2/mPGES-1, HEK-COX-2-10aa-mPGES-1 and HEK-mPGES-1 using high performance liquid chromatography (HPLC)

Activities of the synthases were determined by monitoring metabolites of [^{14}C]-AA for HEK-COX-2/mPGES-1, HEK-COX-2-10aa-mPGES-1, and [^3H]-PGH₂ for HEK-mPGES-1. [^{14}C]-AA or [^3H]-PGH₂ was added to the harvested cells (about 2.2×10^6 cells) in a total reaction volume of 0.2mL, and incubated at room temperature for 5 minutes. For the competition assays between the hot [^3H]-PGH₂ and the cold true substrate PGH₂ (Cold PGH₂) or AA, 0.0125 μCi of [^3H]-PGH₂ and increasing concentration of cold PGH₂ or AA were added to the harvested cells. The reaction was stopped by adding 0.2 mL of Buffer A (containing 0.1% acetic acid and 30% acetonitrile). Subsequent to centrifugation at 13000 rpm for 10 minutes, the supernatant was collected and loaded onto a reverse phase C18 column (Varian Microsorb- MV 100-5, 4.6 mm $\times$ 250 mm). The sample was run with the buffer A with a gradient from 35 to 100% of acetonitrile for 40 minutes with a flow rate 1.0 mL/min. The metabolite profile was obtained directly by a flow scintillation analyzer (Packard 150TR).

2.4. Docking of AA or PGH₂ with mPGES-1

The preparation, protomol generation and docking were performed using SYBYL-X 2.1 (Certara). A crystal structure of mPGES-1 (PDB # 4AL0) was used as a docking model. The structures of AA and PGH₂ were downloaded from PubChem (AA: PubChem CID # 466880, PGH₂: PubChem CID # 445049), and prepared by using the Concord program, computing charges (Huckel), and minimizing energy. The protomol was generated by a residues method. The active site residues were chosen based on the crystal structure information¹⁶ with a

threshold factor of 0.5 Å and a bloat of 0 Å. Docking was carried out with Surflex-Dock Geom (SFXC) mode¹⁷⁻²⁰.

2.5. Site-directed mutageneses of mPGES-1 mutants

cDNAs of the mPGES-1 mutants (R67A, R67K, R70A and R70K) were constructed using a standard PCR method (Figure 1A) as described previously²¹ with their primer set (Table 1). The constructed pcDNA3.1(+)/mPGES-1 wild type and pcDNA3.1(+)/mPGES-1 mutant's plasmids were prepared using Midiprep kit (Qiagen) for transfection into HEK293 cells. The expressions of the mutants were confirmed by Western blot (Figure 1B).

2.6. Binding assay

1.1×10^6 cells were used for the assay. The cells were harvested and transferred into 1.5 mL microcentrifuge tubes. After centrifuging the cells at 1250rpm for 3 minutes, extra medium were removed to set total volume of 100μL. [¹⁴C]-AA (57.1mCi / mmol) was added to the microcentrifuge tubes, and then incubated for 30 minutes at room temperature. The cells were then washed with Dulbecco's modified Eagle's medium three times. Anhydrous Ether (Avantor Performance Materials, Inc) was used to extract lipids in the cells. After air-drying the samples, 250 uL of Buffer A was added to the tubes. The samples were injected and loaded onto a reverse phase C18 column (Varian Microsorb- MV 100-5, 4.6 mm × 250 mm). The sample was run with the buffer A with a gradient from 35 to 100% of acetonitrile for 40 minutes with a flow rate 1.0 mL/min. The metabolite profile was obtained directly by a flow scintillation analyzer (Packard 150TR).

2.7. Statistical analysis

Statistical analysis was performed and shown as the mean $\pm$ the standard deviation using GraphPad Prism 6. Analysis of Variance (ANOVA) was used to compare 2 or more groups. $p < 0.05$ was considered significant.

3. Results

3.1. Establishing a cell line co-expressing COX-2 and mPGES-1 to mimic inflammatory environment for characterization of the two-phase activity of the endogenous omega-6 fatty acid, AA

The three subtypes of PGE₂ synthases, mPGES-1, mPGES-2, and cPGES, share COX-produced PGH₂ as their substrate for the biosynthesis of PGE₂. However, only PGE₂ produced by the inducible mPGES-1 is considered as a major inflammatory PGE₂. Recently, we have reported that COX-2 and mPGES-1 induced by inflammatory factors are organized into an active complex-like configuration in ER membrane to dominate the COX-2-produced PGH₂ ⁵. It explains how inflammation could efficiently be caused by the co-expressed COX-2 and mPGES-1 in tissues. In this study, we co-expressed wild types of human COX-2 and mPGES-1 in HEK293 cells (HEK293-COX-2/mPGES-1) by cDNA transfection to mimic inflammatory cells ²². The expressions of COX-2 and mPGES-1 in the cells were confirmed by Western blot (Figure 2A). The molecular ratio of the expressed mPGES-1 was greater than COX-2 (Figure 2A), and described in a schematic presentation (Figure 2B). The activities of the co-expressed COX-2 and mPGES-1 that catalyzed the added [¹⁴C]-AA (2 μ M) to [¹⁴C]-PGE₂ was identified by an assay that profiles [¹⁴C]-AA metabolites using a HPLC-scintillation analyzer (Figure 2C). These indicate that the cell line could be used to characterize the regulatory effects of the endogenous

omega-6 fatty acid, AA, on PGE₂ biosynthesis. Using a dose-response assay, two phases of AA activities in regulating inflammatory PGE₂ biosynthesis were clearly observed in the HEK293-COX-2/mPGES-1 cells (Figure 2D). In the first phase, using low concentration of [¹⁴C]-AA [$< 2 \mu\text{M}$ (Low- μM)], the cells produced linearly increasing amounts of [¹⁴C]-PGE₂ (Figure 2D). This result was expected because [¹⁴C]-AA was used as a substrate for COX-2. However, with increasing [¹⁴C]-AA levels from $2 \mu\text{M}$ to $10 \mu\text{M}$ (High- μM), [¹⁴C]-PGE₂ production was dramatically inhibited (Figure 2D). This inhibitory effect is a functionally opposite activity of the first phase. This suggests that High- μM AA could be involved in downregulating inflammatory PGE₂ biosynthesis to self-resolve inflammation in the cells, which has not been clearly characterized previously. The High- μM AA could directly bind to the active site of mPGES-1 to competitively block the conversion of COX-2-produced PGH₂ to PGE₂. Nevertheless, the involvement of other PGE₂ synthases, mPGES-2 and cPGES, on the PGE₂ production from COX-2-produced PGH₂ in the HEK293 cells needs to be excluded before reaching this conclusion.

3.2. Identification of two-phase activity of AA using SCEC, COX-2-10aa-mPGES-1, mimicking the complex-like configurations of inflammatory COX-2 and mPGES-1 on the ER membrane that excluded the involvement of other non-inducible PGE₂-synthases.

We have found that HEK293 cells transfected with cDNA of COX-2 alone could produce [¹⁴C]-PGE₂ (Figure 3A) even in the absence of inducible mPGES-1. This indicates that the endogenous non-inducible cPGES and mPGES-2 presenting in the cells could also catalyze COX-2-produced PGH₂ as their substrate to produce PGE₂. To identify the specificity of two-phase activity of AA targeting both COX-2 and mPGES-1, it is important to establish a specific assay system to determine the activities of COX-2 and only mPGES-1 in a condition of

excluding the involvement of cPGES and mPGES-2 in the HEK293 cells. Recently, an active SCEC, COX-2 linked mPGES-1 (COX-2-10aa-mPGES-1) with a minimal separation distance [10 amino acid residues (10aa), approximately 14Å between COX-2 and mPGES-1], have been created²². The SCEC allows the COX-2-domain produced PGH₂ to quickly transport to the active site of the mPGES-1 domain within a single polypeptide chain (Figure 3B). The SCEC could prevent the catalysis of COX-2-produced PGH₂ by other COX-downstream synthases, mPGES-2 and cPGES, because they are further away from the COX-2 active site as compared to the mPGES-1 domain in the SCEC⁵ (Figure 3B). This successful enzyme engineering has allowed us to specifically characterize the activities of an omega-6 fatty acid, AA, on the inflammatory PGE₂ biosynthesis. In addition, we have also found that it is very difficult to control the co-expression of recombinant COX-2 and mPGES-1 at 1:1 ratio in cells. As shown in Figure 2A and 2B, HEK293-COX-2/mPGES-1 cells used for the assay expressed more mPGES-1 than COX-2. This uneven co-expression could become an additional factor to affect the characterization of the two-phase activity of AA. However, the created SCEC, COX-2-10aa-mPGES-1, has an ideal molecular ratio of 1:1 for the expressed COX-2 and mPGES-1 (Figure 3C). It allows us to study monomeric activities for COX-2 and mPGES-1 without interferences from other molecules. Using the HEK293 cells stably expressing the SCEC, COX-2-10aa-mPGES-1 (HEK293-COX-2-10aa-mPGES-1, Figure 3D), which was previously characterized by our group²², as a specific assay target, the two phases of AA activities in regulating inflammatory PGE₂ production were further observed (Figure 3E). The SCEC used Low-μM of [¹⁴C]-AA (<2 μM) to produce [¹⁴C]-PGE₂. However, with increasing [¹⁴C]-AA to High-μM (2-10 μM), the [¹⁴C]-PGE₂ production was dramatically inhibited (Figure 3E).

3.3. Identification of the High- μ M AA directly binding to the active site of mPGES-1

The second phase activity of AA inhibiting mPGES-1 was further confirmed using mPGES-1. The recombinant human mPGES-1 was expressed in HEK293 cells by cDNA transfection, and identified by Western blot (Figure 4A). Using HEK-mPGES-1 as a direct target and [3 H]-PGH₂ as a direct substrate, the conversion of [3 H]-PGH₂ to [3 H]-PGE₂ produced by mPGES-1 were compared in the presence (Figure 4B, top panel) and absence (Figure 4B, bottom panel) of unlabeled High- μ M AA (10 μ M). In the presence of High- μ M AA, the mPGES-1 activity was inhibited by more than 90% (Figure 4B bottom panel). In addition, the competition assays between the hot [3 H]-PGH₂ and the cold true substrate PGH₂ (Cold PGH₂) or AA showed almost identical IC₅₀ values for [3 H]-PGE₂ production (8.17 μ M for Cold PGH₂ and 6.56 μ M for AA), and complete inhibition at 100 μ M (Supplemental Figure 1). These experiments have thus demonstrated that High- μ M AA could bind directly to the substrate-binding site of mPGES-1, and validated the observations that endogenous AA has two phase activities in regulating inflammatory PGE₂ biosynthesis in the studies using the co-expression of COX-2 and mPGES-1, and the SCEC, COX-2-10aa-mPGES-1.

3.4. Testing the specificity of High- μ M AA binding to mPGES-1 in primary cultured-cells

To further address the specificity of AA, in which the inhibition of inflammatory mPGES-1 without affecting non-inducible cPGES and mPGES-2 for the production of a basal level PGE₂, we analyzed the effects of AA on PGE₂ production using the previously characterized adipocytes, which lack mPGES-1, but contain cPGES and mPGES-2²³. In this paper, adipocytes were cultured (Figure 5A), and then profiled for the PGE₂ production under High- μ M AA (10 μ M) condition. The cells converted 10 μ M [14 C]-AA into [14 C]-PGE₂ with very

little other prostanoids (Figure 5B). This result indicates that AA does not inhibit cPGES and mPGES-2 or their production of a basal level of PGE₂. In addition, we were interested in determining whether High- μ M AA has any effect on inhibiting other COX downstream enzymes, such as PGIS. Using HEK293 cells co-expressing COX-2 and PGIS (COX-2/PGIS), or COX-2-10aa-PGIS, as well as [¹⁴C]-AA as their substrate, only a single phase increase in [¹⁴C]-6-keto-PGF_{1 α} (degraded from [¹⁴C]-PGI₂) production with an increasing concentration of [¹⁴C]-AA up to 27.5 μ M was observed (Figure 5C). This clearly demonstrates that High- μ M AA inhibits mPGES-1, but has no effect on PGIS activity or its production of the vascular protector, PGI₂, in the primary cultured-fat cells.

3.5. Identification of the key residues of AA binding site on mPGES-1 using X-ray structure-based docking

Docking of the high resolution X-ray structure of mPGES-1 with AA and the enzyme substrate PGH₂ has revealed that both AA and PGH₂ could bind to the identical pocket of mPGES-1 with similar affinities (Figure 6A). The total binding scores are 8.98 and 8.54 for AA and PGH₂, respectively. In addition, R67 and R70 of mPGES-1 bound to a carboxylic acid group of AA and PGH₂ through identical H-bond donors and acceptors, in which R67 and R70 could be the key residues for making salt bridges between the compounds and mPGES-1 (Figure 6B). These data provided us with important information to select the residues for a site-directed mutagenesis approach and expression using a recombinant enzyme.

3.6. Validation of key residues R67 and R70 of mPGES-1 binding to AA by site-directed mutagenesis

To further explore the binding properties of mPGES-1, four cDNAs of mPGES-1 mutants, R67A, R67K, R70A and R70K were created by site-directed mutagenesis using PCR (Figure 1A). The mutants were stably expressed in HEK293 cells through cDNA transfection and G418 screening (Figure 1B). Comparing to the wild type mPGES-1, all of the mutants lost 40-100% of binding to [¹⁴C]AA using untransfected HEK cells as a background (Figure 6C). Interestingly, the Arg-to-Lys mutants, which conserve the positive charge of the side chain, exhibited a reduced AA binding capacity. Therefore, it could indicate that the active residues in the mPGES-1 active site responsible for the binding to the ligand could be involved in several intermolecular intersections, such as charge, hydrogen bond, hydrophobic and steric hindrance. The fact that Arg-to-Lys mutants could not restore the enzyme activity suggests that in addition to the positive charge being important to the binding activity, the difference of the other intermolecular interactions between the Arg and Lys residues are also involved in the formation of a substrate binding site. Further computational studies using mPGES-1 R67K and R70K models have also suggested the hydrogen donors on the side of Arg could not be mimicked by the side chain of Lys (Figure 7). This data led us to conclude that R67 and R70 are key residues binding to AA, and the cellular lipid molecule, AA, has two phase activities directly involved in controlling inflammation *via* concentration-dependent mechanisms, activation of COX-2 and inhibition of mPGES-1 to regulate cellular inflammatory PGE₂ biosynthesis.

4. Discussion

For decades, it has been recognized that AA can bind to three enzymes, COX, Lipoxygenase and cytochrome P450, to be metabolized to bioactive compounds, which directly involve in mediating pathophysiological processes, such as, hemostasis, blood flow, and

inflammation. In this study, characterization of the concentration-dependent two-phase activity of AA on inflammatory PGE₂ biosynthesis and the identification of the key residues, R67 and R70 of mPGES-1 binding to AA, led us to propose an additional pathway for AA, targeting mPGES-1. This finding has provided important impacts on further understanding molecular mechanisms of the related diseases mediated by AA metabolites through COX-2/mPGES-1 pathway. It showed potential that the inducible phospholipase A2 /COX-2/mPGES-1 pathway can causes inflammation to repair tissues or damaged tissues, but also has a self-controlling ability to limit the repair and damage. It can be speculated that when cells are stimulated by inflammatory factors, AA starts to release out from the membrane through activation of phospholipase A2, and COX-2 and mPGES-1 induced their expression. At the beginning, the low concentration of AA is used as a substrate for COX-2 to produce PGH₂, which is immediately passed to mPGES-1 to produce the end product of inflammatory PGE₂. The produced PGE₂ further stimulates tissue inflammation and activates more phospholipase A2 to release more AA for producing more PGE₂. However, when the concentration of AA reached to μ M level, AA starts to bind to the mPGES-1 substrate pocket to reduce inflammatory PGE₂ production and limits tissue repair and damage. The reduced PGE₂ stops the stimulation of phospholipase A2, and then reduces AA release from the membrane while the consumption of AA by COX-2 is not stopped. This leads to the reduction of the AA concentration to Low- μ M level. At the low concentration, AA leaves from the substrate binding pocket of mPGES-1, leading to a recovery of inflammatory PGE₂ synthesis by mPGES-1, and starts a new circle to regulate PGE₂ biosynthesis (Figure 8). Thus, endogenous AA has dual activities, as inflammatory and anti-inflammatory.

The HEK-COX-2/mPGES-1 and HEK-COX-2-10aa-mPGES-1 cell assays both showed two phase activities. Interestingly, the inhibition arrested at about 50%, which is not the case for typical competitive types of enzyme inhibition. However, this incomplete inhibitory effect is very reasonable since the production and inhibition of PGE₂ biosynthesis happens at the same time - the arachidonic acid as a substrate for COXs and as an inhibitor for mPGES-1 during inflammatory PGE₂ biosynthesis inside cells. In fact, in HEK-mPGES-1 cells, the competition assay using hot [³H]-PGH₂ and cold PGH₂ or AA showed completely inhibited [³H]-PGE₂ production at 100μM. It should be noted that the higher concentration (>10μM) of AA is toxic to the cells, and it does not have much meaning to the cellular study.

The above model is not only for presumption of a self-control inflammatory process, but it might also help reveal unknown mechanisms of how acute and chronic inflammation occurs when PGE₂ is involved. For example, when tissues and cells receive severe injury, it triggers an acute inflammation, and a large amount of PGE₂ is produced by the acute activation of phospholipase A2/COX-2/mPGES-1. The produced PGE₂ further stimulates a large AA release, and then inhibits mPGES-1 to quickly reduce PGE₂ biosynthesis, which contributes to a recovery from inflammation. Thus, the acute inflammation only happens for a short time. However, in chronic inflammation, PGE₂ produced by COX-2/mPGES-1 is not high enough to stimulate AA release, and thereby keeps AA at a moderate level, which cannot inhibit mPGES-1. This could be a reason that the chronic inflammatory process lasts long and shows less ability to self-control. The implication of this is that the addition of exogenous PUFAs, such as linoleic acid, which can be converted into AA in cells, may also be able to inhibit mPGES-1 to reduce chronic inflammatory PGE₂ biosynthesis. This could explain the mechanisms of the results obtained by animal and clinical studies that increasing the intake of omega-6 PUFAs to at least 5-10% of

energy could reduce the risk of CHD, and that higher intake could be even more beneficial to reduce CHD ⁶.

Chronic vascular inflammation is one of the major pathological processes leading to CHD. This study and findings of the two-phase activity of omega-6 PUFA, AA, could give an explanation that the extra amount of PUFA, which contains a large amount of linoleic acid, could be converted into High- μ M AA (Figure 8) to inhibit mPGES-1 activity. This could be one of the reasons why taking a large amount of omega-6 PUFA could reduce the vascular inflammation and CHD.

This study has also provided another explanation of NSAID activity on treating inflammation. NSAIDs have a potent anti-inflammatory activity. Beside inhibition of COX-2 activity to reduce PGE₂ production, NSAIDs can also increase cellular AA concentration as a result of inhibiting COX-2 activity ^{24–27}. The increased AA level could be an important factor to inhibit mPGES-1 activity and to reduce inflammatory PGE₂ production. Additionally, clinical studies have indicated that NSAIDs and PUFAs may reduce cancer growth ^{28–31}. The molecular mechanisms are not settled yet. However, it is known that chronic inflammation mediated by PGE₂ could promote cancer growth ^{32–34}. The finding of AA inhibiting mPGES-1 could explain the mechanisms of the benefit coming from taking NSAIDs and omega-6 fatty acid to reduce cancer risk. Our unique hybrid enzyme, COX-2-10aa-mPGES-1, which mimics the complex-like configuration of COX-2 and mPGES-1, has increased the impact on screening more specific compounds inhibiting mPGES-1 by excluding the involvement of cPGES and mPGES-2, and other prostanoid synthases. The identified key residues for AA binding to mPGES-1 have provided important clues to understand the mechanism of anti-inflammatory action produced by

PUFAs. They contain a similar carboxylic acid group that is seen in AA, which may bind to R67 and R70 to reduce mPGES-1 activity and inhibit inflammatory PGE₂ biosynthesis.

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Figure Legends

Table 1. Primers used for a standard PCR to construct cDNAs of the mPGES-1 mutants.

Figure 1. A) Agarose gel electrophoresis of PCR for constructing cDNAs of mPGES-1 mutants. pcDNA: pcDNA3.1 (+)/mPGES-1 wild type. B) Western blot for mPGES-1 wild type and mutants expressed in HEK293. Untransfected HEK293 cells were used as a control.

Figure 2. A) Western blot analysis for HEK293 cells stably co-expressing COX-2 and mPGES-1 (HEK293-COX-2/mPGES-1) by cDNA transfection and G418 screening. B) Model showing the expressed molecular ratio of COX-2 and mPGES-1. Expression of mPGES-1 is higher than COX-2. C) Effective biosynthesis of inflammatory PGE₂ by the HEK293-COX-2/mPGES-1 cells. Almost all of the added 2 μ M of [¹⁴C]-AA was converted to [¹⁴C]-PGE₂. D) Concentration-dependent two-phase activity of AA of increasing (<2 μ M) and decreasing (>2 μ M) PGE₂ biosynthesis were found.

Figure 3. A). [¹⁴C]-AA metabolites produced by HEK293, indicating that the endogenous cPGES and mPGES-2 constitutively expressed in HEK293 cells could also catch the COX-2-produced [¹⁴C]-PGH₂ to catalyze into [¹⁴C]-PGE₂ in the HEK293 cells. B) Putative model of COX-2-10aa-mPGES-1 expressed on the ER membrane. C) Model showing the expressed molecular ratio of COX-2 and mPGES-1 is 1 to 1. D) Western blot analysis of the expressed SCEC, COX-2-10aa-mPGES-1, and the co-expressed COX-2 and mPGES-1 in HEK293 cells. E) Dose-response curves for the inflammatory PGE₂ production catalyzed by COX-2 domain and mPGES-1 domain within the single polypeptide chain by increasing amounts of [¹⁴C]-AA.

Figure 4. A) Western blot analysis for the mPGES-1 expressed in HEK293 cells. B) HPLC profile analysis of the effects of AA on mPGES-1 activity converting the direct substrate [^3H]-PGH₂ to [^3H]-PGE₂.

Figure 5. A) Cultured mouse adipocytes at day 1 and day 10. B) The cells were able to convert High- μM (10 μM) [^{14}C]-AA to [^{14}C]-PGE₂. C) Single phase of increasing PGI₂ ([^{14}C]-6-Keto-PGF_{1 α}) production with the increasing concentrations of [^{14}C]-AA was observed [23].

Figure 6. A) Docking of AA and PGH₂ with the X-ray structure of mPGES-1. AA and PGH₂ share the binding pocket on mPGES-1. B) H-bonds and salt bridges between R67/R70 of the mPGES-1 and the carboxylic groups of AA and PGH₂ are shown. C) Binding of [^{14}C]-AA to wild type and mPEGS-1 mutants. R67 and R70 mutants lost activity bound to [^{14}C]-AA are shown.

Figure 7. A decreased number of hydrogen bonds in mPGES-1 mutants shown in superimposed models. mPGES-1 WT (R67/R70) and mPGES-1 R67K (A) and mPGES-1 WT and mPGES-1 R70K (B) were superimposed. Residues for the results from mPGES-1 WT docking are shown by atom type (H = cyan, O = red, N = blue, and C = white). Residues for the results from mPGES-1 R67K and R70K dockings are shown in purple. Hydrogen bonds that were detected by Sybyl-X 2.1 were shown in dash lines, and numbers of hydrogen bonds are shown in the figures. Hydrogen bonds marked with an arrow (light orange) were undetected in mutant dockings.

Figure 8. Model of the mechanisms of the concentration-dependent two-phase activity of AA involved in regulating the tissue inflammation and resolution. When cells are stimulated by inflammatory factors (IF), cPLA₂ is activated to release AA. The induced COX-2 and mPGES-1, which undergo a post-translational arrangement into a complex-like (<14Å) configuration on ER membrane, will effectively shift AA metabolism from a balanced PGE₂ production to an unbalanced (excessive) PGE₂ production to mediate vascular inflammation. The excessive PGE₂ becomes additional IF to further stimulate cPLA₂ releasing a higher level of AA (>2μM), which directly binds to mPGES-1 to inhibit the PGE₂ production, and thereby helps resolve the tissue inflammation in vascular cells.

References

- (1) Parent, J., Chapdelaine, P., and Fortier, M. A. (2005) Molecular cloning and tissue distribution of microsomal-1 and cytosolic prostaglandin e synthases in macaque. *Prostaglandins Other Lipid Mediat.* 78, 27–37.
- (2) Parent, J., and Fortier, M. A. (2005) Expression and contribution of three different isoforms of prostaglandin E synthase in the bovine endometrium. *Biol. Reprod.* 73, 36–44.
- (3) Schneider, A., Zhang, Y., Zhang, M., Lu, W. J., Rao, R., Fan, X., Redha, R., Davis, L., Breyer, R. M., Harris, R., Guan, Y., and Breyer, M. D. (2004) Membrane-associated PGE synthase-1 (mPGES-1) is coexpressed with both COX-1 and COX-2 in the kidney. *Kidney Int.* 65, 1205–1213.
- (4) Hansen, K., Varvas, K., Järving, I., and Samel, N. (2014) Novel membrane-associated prostaglandin E synthase-2 from crustacean arthropods. *Comp. Biochem. Physiol. Part - B Biochem. Mol. Biol.* 174, 45–52.
- (5) Akasaka, H., So, S.-P., and Ruan, K.-H. (2015) Relationship of the Topological Distances and Activities between mPGES-1 and COX-2 versus COX-1: Implications of the Different Post-Translational Endoplasmic Reticulum Organizations of COX-1 and COX-2. *Biochemistry* 54, 3707–3715.
- (6) Harris, W. S., Mozaffarian, D., Rimm, E., Kris-Etherton, P., Rudel, L. L., Appel, L. J., Engler, M. M., Engler, M. B., and Sacks, F. (2009) Omega-6 fatty acids and risk for cardiovascular disease: A science advisory from the American Heart Association nutrition subcommittee of the council on nutrition, physical activity, and metabolism; council on cardiovascular nursing; and council on epidem. *Circulation* 119, 902–907.
- (7) Levick, S. P., Loch, D. C., Taylor, S. M., and Janicki, J. S. (2007) Arachidonic Acid

Metabolism as a Potential Mediator of Cardiac Fibrosis Associated with Inflammation. *J.*

Immunol. 178, 641–646.

(8) Funk, C. D. (2001) Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* 294, 1871–1875.

(9) Caughey, G. E., Cleland, L. G., Penglis, P. S., Gamble, J. R., and James, M. J. (2001) Roles of Cyclooxygenase (COX)-1 and COX-2 in Prostanoid Production by Human Endothelial Cells: Selective Up-Regulation of Prostacyclin Synthesis by COX-2. *J. Immunol.* 167, 2831–2838.

(10) Penglis, P. S., Cleland, L. G., Demasi, M., Caughey, G. E., and James, M. J. (2000) Differential regulation of prostaglandin E2 and thromboxane A2 production in human monocytes: implications for the use of cyclooxygenase inhibitors. *J. Immunol.* 165, 1605–1611.

(11) Huwiler, A., and Pfeilschifter, J. (2009) Lipids as targets for novel anti-inflammatory therapies. *Pharmacol. Ther.* 124, 96–112.

(12) Hwang, S. H., Weckslar, A. T., Wagner, K., and Hammock, B. D. (2013) Rationally Designed Multitarget Agents Against Inflammation and Pain. *Curr. Med. Chem.* 20, 1783–1799.

(13) Kamei, D., Murakami, M., Nakatani, Y., Ishikawa, Y., Ishii, T., and Kudo, I. (2003) Potential role of microsomal prostaglandin E synthase-1 in tumorigenesis. *J. Biol. Chem.* 278, 19396–19405.

(14) Murakami, M., Naraba, H., Tanioka, T., Semmyo, N., Nakatani, Y., Kojima, F., Ikeda, T., Fueki, M., Ueno, A., Oh-ishi, S., and Kudo, I. (2000) Regulation of Prostaglandin E2 Biosynthesis by Inducible Membrane-associated Prostaglandin E2 Synthase That Acts in Concert with Cyclooxygenase-2. *J. Biol. Chem.* 275, 32783–32792.

(15) Ruan, K.-H., Deng, H., and So, S. P. (2006) Engineering of a protein with cyclooxygenase and prostacyclin synthase activities that converts arachidonic acid to prostacyclin. *Biochemistry*

45, 14003–14011.

(16) Sjogren, T., Nord, J., Ek, M., Johansson, P., Liu, G., and Geschwindner, S. (2013) Crystal structure of microsomal prostaglandin E2 synthase provides insight into diversity in the MAPEG superfamily. *Proc. Natl. Acad. Sci.* 110, 3806–3811.

(17) Dutta Gupta, S., Snigdha, D., Mazaira, G. I., Galigniana, M. D., Subrahmanyam, C. V. S., Gowrishankar, N. L., and Raghavendra, N. M. (2014) Molecular docking study, synthesis and biological evaluation of Schiff bases as Hsp90 inhibitors. *Biomed. Pharmacother.* 68, 369–376.

(18) Wang, R., Lu, Y., Fang, X., and Wang, S. (2004) An extensive test of 14 scoring functions using the PDBbind refined set of 800 protein-ligand complexes. *J. Chem. Inf. Comput. Sci.* 44, 2114–2125.

(19) Rarey, M., Kramer, B., and Lengauer, T. (1997) Multiple automatic base selection: protein-ligand docking based on incremental construction without manual intervention. *J. Comput. Aided. Mol. Des.* 11, 369–384.

(20) Wang, R., Lu, Y., and Wang, S. (2003) Comparative evaluation of 11 scoring functions for molecular docking. *J. Med. Chem.* 46, 2287–2303.

(21) Chillar, A., Wu, J., So, S. P., and Ruan, K.-H. (2008) Involvement of non-conserved residues important for PGE2 binding to the constrained EP3 eLP2 using NMR and site-directed mutagenesis. *FEBS Lett.* 582, 2863–2868.

(22) Ruan, K.-H., Cervantes, V., and So, S. P. (2009) Engineering of a novel hybrid enzyme: An anti-inflammatory drug target with triple catalytic activities directly converting arachidonic acid into the inflammatory prostaglandin E2. *Protein Eng. Des. Sel.* 22, 733–740.

(23) Ruan, K.-H., Mohite, A., So, S.-P., and Ruan, C.-H. (2013) Establishing novel prostacyclin-synthesizing cells with therapeutic potential against heart diseases. *Int. J. Cardiol.* 163, 163–169.

- (24) Fosslien, E. (2005) Review : Cardiovascular Complications of Non-Steroidal Anti-Inflammatory Drugs 35, 347–385.
- (25) Sagi, S. A., Weggen, S., Eriksen, J., Golde, T. E., and Koo, E. H. (2003) The Non-cyclooxygenase Targets of Non-steroidal Anti-inflammatory Drugs, Lipxygenases, Peroxisome Proliferator-activated Receptor, Inhibitor of B Kinase, and NF B, Do Not Reduce Amyloid 42 Production. *J. Biol. Chem.* 278, 31825–31830.
- (26) Giardina, C., and Inan, M. S. (1998) Nonsteroidal anti-inflammatory drugs, short-chain fatty acids, and reactive oxygen metabolism in human colorectal cancer cells. *Biochim. Biophys. Acta* 1401, 277–288.
- (27) Ghosh, R., Alajbegovic, A., and Gomes, A. V. (2015) NSAIDs and Cardiovascular Diseases: Role of Reactive Oxygen Species. *Oxid. Med. Cell. Longev.* 2015, 1–25.
- (28) de Lorgeril, M., and Salen, P. (2012) New insights into the health effects of dietary saturated and omega-6 and omega-3 polyunsaturated fatty acids. *BMC Med.* 10, 50.
- (29) Pizato, N., Bonatto, S., Yamazaki, R. K., Aikawa, J., Nogata, C., Mund, R. C., Nunes, E. a, Piconcelli, M., Naliwaiko, K., Curi, R., Calder, P. C., and Fernandes, L. C. (2005) Ratio of n6 to n-3 fatty acids in the diet affects tumor growth and cachexia in Walker 256 tumor-bearing rats. *Nutr. Cancer* 53, 194–201.
- (30) Piazuelo, E., and Lanas, A. (2015) NSAIDS and gastrointestinal cancer. *Prostaglandins Other Lipid Mediat.* 120, 91–96.
- (31) Liggett, J. L., Zhang, X., Eling, T. E., and Baek, S. J. (2014) Anti-tumor activity of non-steroidal anti-inflammatory drugs: Cyclooxygenase-independent targets. *Cancer Lett.* 346, 217–224.
- (32) Greenhough, A., Smartt, H. J. M., Moore, A. E., Roberts, H. R., Williams, A. C., Paraskeva,

C., and Kaidi, A. (2009) The COX-2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. *Carcinogenesis* 30, 377–386.

(33) Xia, D., Wang, D., Kim, S.-H., Katoh, H., and DuBois, R. N. (2012) Prostaglandin E2 promotes intestinal tumor growth via DNA methylation. *Nat. Med.* 18, 224–226.

(34) Nakanishi, M., and Rosenberg, D. W. (2013) Multifaceted roles of PGE2 in inflammation and cancer. *Semin. Immunopathol.* 35, 123–137.

Figure 1

A

B

Figure 2

Figure 3

A

B

C

COX-2-10aa-mPGES-1

D

E

Figure 4

Figure 5

Figure 6

A AA R70

Total score: 8.98

R67 PGH₂

Total score: 8.54

B

C

Figure 7

Figure 8

Table 1. Primers used for a standard PCR to construct cDNAs of the mPGES-1 mutants.

Mutation	Forward/Reverse	Sequence
mPGES-1 R67A	Forward	CCGACGTGGAAGCCTGCCTCAGGGC
	Reverse	GCCCTGAGGCAGGCTTCCACGTCGG
mPGES-1 R67K	Forward	GACCCCGACGTGGAAAAGTGCCTCAGGGCCCAC
	Reverse	GTGGGCCCTGAGGCACTTTTCCACGTCGGGGTC
mPGES-1 R70A	Forward	GAACGCTGCCTCGCCGCCACCGGAAC
	Reverse	GTTCCGGTGGGCGGCGAGGCAGCGTTC
mPGES-1 R70K	Forward	GAACGCTGCCTCAAGGCCACCGGAAC
	Reverse	GTTCCGGTGGGCCTTGAGGCAGCGTTC