The Key Residue within the Second Extracellular Loop of Human EP3 Involved in Selectively Turning Down PGE₂- and Retaining PGE₁-Mediated Signaling in Live Cells

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ABBREVIATIONS

arachidonic acid, AA; cyclooxygenase-1 and -2, COX-1 and -2, respectively; cytosolic PGES, cPGES; dihomo- γ -linolenic acid, DGLA; prostaglandin E receptor, EP; extracellular loop, eLP; gastrointestinal, GI; G-protein coupled receptors, GPCRs; human embryonic kidney, HEK; human prostaglandin E receptor, hEP; human thromboxane receptor, hTP; microsomal PGE synthase-1 and -2, mPGES-1 and -2, respectively; nonsteroidal anti-inflammatory drugs, NSAIDs; nuclear magnetic resonance, NMR; prostaglandin, PG; prostaglandin D₂, PGD₂; prostaglandin F₂, PGF₂; prostacyclin or prostaglandin I₂, PGI₂; prostacyclin receptor, IP; Prostaglandin E₁ and E₂, PGE₁ and PGE₂, respectively; Protein Data Bank, PDB; phospholipase C, PLC; protein kinase A, PKA; protein kinase C α , (PKC α), thromboxane A₂ receptor, TP; thromboxane A₂, TXA₂; transmembrane, TM; unsaturated fatty acids, UFAs

ABSTRACT

Key residues and binding mechanisms of PGE₁ and PGE₂ on prostanoid receptors are poorly understood due to the lack of X-ray structures for the receptors. We constructed a human EP3 (hEP3) model through integrative homology modeling using the X-ray structure of the β_2 -adrenergic receptor transmembrane domain and NMR structures of the thromboxane A2 receptor extracellular loops. PGE₁ and PGE₂ docking into the hEP3 model showed differing configurations within the extracellular ligand recognition site. While PGE₂ could form possible binding contact with S211, PGE₁ is unable to form similar contacts. Therefore, S211 could be the critical residue for PGE₂ recognition, but is not a significant for PGE₁. This prediction was confirmed using HEK293 cells transfected with S211L cDNA. The S211L cells lost PGE₂ binding and signaling. Interestingly, the S211L cells retained PGE₁-mediated signaling. It indicates that S211 within the second extracellular loop is a key residue involved in turning down PGE₂ signaling. Our study provided information that S211L within EP3 is the key residue to distinguish PGE₁ and PGE₂ binding to mediate diverse biological functions at the initial recognition step. The S211L mutant could be used as a model for studying the binding mechanism and signaling pathway specifically mediated by PGE₁.

1. Introduction

Prostaglandin (PG) E₁ and E₂ (PGE₁ and PGE₂, respectively) are synthesized through the cyclooxygenase (COX) pathway by coupling reactions of COX-1 or COX-2 and PGE₁/E₂ synthases (PGES) using 20-carbon ω -6 fatty acids of dihomo- γ -linolenic acid (DGLA) and arachidonic acid (AA) as substrates, respectively¹⁻⁴. Three PGES, noninducible types of cytosolic PGES (cPGES)^{5,6} and microsomal PGES-2 (mPGES-2)^{6,7}, and an inducible type of microsomal PGES-1 (mPGES-1)^{5,6,8,9} have been cloned and characterized previously. PGE₂ synthesized by the inducible mPGES-1 is one of the major mediators for inflammatory diseases, such as arthritis^{10,11}, vascular inflammation^{12,13}, angiogenesis and some types of cancers¹⁴⁻¹⁸. However, PGE₁ synthesized by the same enzymes has less inflammatory effects, mediating smooth muscle relaxation as well as promoting blood flow¹⁹. PGE₁ and PGE₂ are considered endogenous molecules that competitively bind to EPs²⁰⁻²⁴ as their primary targets, and they could also bind to other prostanoid receptors with different affinities. For example, PGE₁, but not PGE₂, binding to IP receptor ($pK_i = 6.8$) was reported²⁵. However, the key residues and binding mechanisms of prostanoid receptors that discriminate PGE₁ and PGE₂ are poorly understood. A lack of the 3D-structural models has been a major obstacle in prostanoid receptor-ligand binding studies.

PGE₁ and PGE₂ primarily share four receptors, EP1, EP2, EP3 and EP4, to exert differing biological functions through diverse signaling pathways²⁶. It has been reported that EP1 can activate phospholipase C (PLC), protein kinase C α (PKC α) and c-Src^{27,28}. EP2 and EP4 can promote signaling of cAMP/protein kinase A (PKA) and phosphoinositide-3-kinase²⁹. EP3 has the most complex signaling, which primarily couples to G_i, resulting in the inhibition of adenylyl cyclase. However, it has also been reported that secondary transduction of G_s resulting in cAMP production^{26,30,31}, and cellular calcium mobilization mediated through G_q³². The EP3 mediated-calcium signaling *via* G_i was observed in EP3 cDNA-transfected Chinese hamster cells³³. EP3 can also activate the Ras signaling pathway³⁴. Therefore, it becomes available to use calcium or cAMP signaling for characterization of EP3 activation in cells^{32,33}. In particular, the cellular calcium mobilization could be developed into an assay to instantly observe the EP3 signaling in live cells. The signaling pathways for EP3 have been identified, and the PGE₁ and PGE₂

affinities on EP3 are indistinguishable³⁵. Therefore, in this study we decided to use EP3 as a target to address the specificity and selectivity of the PGE₁ and PGE₂ binding to prostanoid receptors. Based on X-ray crystal structures of G-protein coupled receptors (GPCRs), it is believed that ligand binding pockets for small molecules exist within transmembrane (TM) domains. However, a TM domain pocket has no impact on determining PGE₁ and PGE₂ selectivity. The residues of the pocket are identical and are also covered by extracellular domains, making them inaccessible in molecule-docking at the initial step to recognize PGE₁ or PGE₂. All of the prostanoids including PGE₂, TXA₂, PGI₂, PGD₂ and PGF₂ have similar chemical structures as they are all derived from the same substrate, arachidonic acid. Understanding how one of the prostanoids is recognized by its target receptors will provide information about activities of other receptors. Previously, we identified the residues and sites within the extracellular loops involved in specific ligand recognition in other prostanoid receptors, including thromboxane A2 receptor (TP)^{36–39}, prostacyclin receptor (IP)^{38,39} and human EP3 (hEP3)⁴⁰. Multiple approaches were utilized, including site-directed mutagenesis, structural determination using solution nuclear magnetic resonance (NMR), and advanced computational skills. This observation is also supported by the results from a mutagenesis study conducted by another group⁴¹. Our research suggests that the specific residues within the second extracellular loops (eLP2) of the prostanoid receptors are important in specific ligand recognition. These observations have helped to elucidate the mechanisms involved in selective ligand recognition in prostanoid receptors especially with one of the most diverse signaling mediated by PGE₁/E₂.

Chemically and structurally, PGE₁ and PGE₂ are almost identical except PGE₂ has an additional double bond, not found in PGE₁³. The role of the double-bond in PGE₂, relative to EP3 receptor recognition and binding, is the focus of this study. We started with mutagenesis to validate the working model of hEP3 constructed with homology modeling using the X-ray structure of the β_2 -adrenergic receptor as the TM domain templates. We then assembled NMR structures of the extracellular fragments of the human thromboxane A2 receptor (TP) as extracellular loop (eLP) templates. Subsequently, we moved on to identify the specific residue, S211 in the eLP2 region involved in “turning down” and

“retain” EP3 to recognize PGE₂. Finally, we found that EP3 S211L mutant cells specifically activated PGE₁-mediated cAMP inhibitory activity and calcium signaling, and failed that of PGE₂ in the live cells. The S211L mutant provided us the first model for studying the binding mechanism and signaling pathway specifically mediated by PGE₁, but not PGE₂ in EP3 receptors. This binding mechanism could be applied to study specific affinities of other prostanoid receptors.

2. Material and methods

2.1. Homology modeling using X-ray structure of β_2 -adrenergic receptor for TM domains of hEP3.

The X-ray crystal structure of the β_2 -adrenergic receptor with a resolution at 2.40 Å (PDB # 2RH1)⁴⁴ was downloaded from Protein Data Bank (PDB), and the 3D structures of the TM domains were extracted using Insight II software on a SIG workstation. These structures were used for homology modeling of the TM domains of the EP3 receptors.

2.2. Homology modeling using NMR structures of TP for extracellular loops for hEP3.

We have solved all of the NMR structures of the constrained peptides mimicking the three eLPs of the human TP receptor⁴⁵. These are saved in our database. The NMR structures were converted to PDB format and then used for the homology modeling of the EP3 eLP domains using Insight II software on a SIG workstation based on accession number P43115-1.

2.3. Docking of PGE₁ and PGE₂ with the wild type and mutant hEP3.

Molecular dynamics and ligand docking were performed with SYBYL-X 2.1 (CERTARA USA, Inc). The S211L mutant EP3 model was constructed by replacing the residues. The 3D structures of PGE₁ and PGE₂ were downloaded from PubChem (PGE₁: CID # 5280723, PGE₂: CID # 5280360). The ligands were prepared by using the Concord program, computing charges (Hückel), and minimizing energy. The EP3 model was prepared by minimizing energy (Powell) and adding hydrogen bonds. The protomol was generated by multichannel methods with a threshold factor of 0.5 Å and a bloat of 0 Å. Docking was carried out with Surflex-Dock Geom (SFXC) mode⁴⁶⁻⁴⁹.

2.4. Site-directed Mutagenesis for Preparation of hEP3 S211L mutant.

EP3 S211L mutant cDNA was constructed using a standard PCR method as described previously⁴². The constructed pcDNA3.1(+)/EP3 wild type and pcDNA3.1(+)/S211L plasmids were prepared using Midiprep kit (Qiagen) for transfection into HEK293 cells.

2.5. Establishing HEK293 cell lines stably expressing recombinant hEP3.

Previously established hEP3 expressing HEK293 cells were used for this study. Briefly, HEK293 cell were transfected with purified cDNA of the pcDNA3.1(+)/EP3 wild type or EP3 S211L with Lipofectamine 2000 method, according to the manufacturer's instructions (Invitrogen). The transfected cells were cultured in the presence of G418 for several weeks to prepare the cell lines stably expressing the recombinant EP3. Western blot was performed as described previously⁴².

2.6. Live cell calcium signaling assay for recombinant hEP3.

The calcium signaling assay was performed on live cells using Fluo-8 AM dye as an indicator. The cells were cultured in 12-well glass-bottom plates, and incubated with Fluo-8 AM dye (excitation 490 nm, emission 525 nm) dissolved in Modified Hank's buffered salt solution (HBSS, without Calcium and Magnesium) containing 10 mM HEPES, pH 7.6, and 0.1% bovine serum albumin (HBSSHB buffer) for 20 minutes. The cells were then washed three times for perturbation with the buffer containing HBSSHB with Probenecid Acid (2.5 mM) and Pluronic F-68 (0.1%), and incubated for an additional 10 minutes. Afterwards, calcium signaling mediated by EP3 was induced by applying PGE₁ or PGE₂ on the live cells. Signaling was monitored using a Nikon Ti-S eclipse microscope (40X objective) and three trials were conducted⁵⁰. The cell viability following the experiment was confirmed by the trypan blue dye exclusion method.

2.7. Determination of EP3 signaling using cAMP assay.

The EP receptors in HEK293 cell lines were analyzed for signal transduction capability mediated by Gs coupling using a cAMP assay according to the manufacture instructions (Amersham Biosciences). The stable cell lines were placed in 96-well plates, and cultured overnight at 37°C in a humidified 5% CO₂ atmosphere in DMEM+ 10% FBS, antibiotics, and antimycotics. The cells were then incubated with 100

pM and 10 nM of PGE₂ and PGE₁ for 10 minutes at 37°C in the culture medium. After the incubation, the medium was removed and the cells were lysed. The intracellular cAMP produced by the individual EP receptors was quantified in the 96-well microplates by Enzyme Immunoassay (EIA) using a cAMP Biotrak EIA System. A set of three experiments were performed. The cyclic AMP in HEK293 cells as controls was compared with that of the EP receptors stimulated by PGE₁ and PGE₂. In order to determine the inhibition of cAMP formation, HEK293 cells expressing EP3 and EP3S211L mutant were cultured overnight in a 48-well plate (about 0.7 x 10⁴ cells/well). The medium was removed and replaced with the medium containing treatment (1μM forskolin only, 1μM forskolin + PGE₁, or 1μM forskolin + PGE₂). The cells were then incubated for 20 minutes at 37 °C. After incubation, the amount of cAMP was measured using a Cyclic AMP EIA Kit (Cayman Chemical, Ann Arbor, MI) by following the manufacturer's instructions.

3. RESULTS

3.1. Establishment of the 3D working model for the wild type hEP3

The conserved seven TM domains of hEP3 were constructed (Figure 1A) by homology modeling using the crystal structure of the β₂-adrenergic receptor as a template⁵¹. The extracellular loops of hEP3 contain a key region involved in the formation of the PGE₁/E₂ ligand recognition site. To create a 3D structural model for hEP3, we constructed extracellular loops of hEP3 by homology modeling obtained from the 3D-NMR structures of the TP extracellular loops⁴⁵, which were successfully used as a structural template for construction of the extracellular loop model of PGI₂ receptor (IP) previously⁴¹. TP, IP and EPs are sister receptors that can bind to other prostanoids with different affinities^{52,53}. It is believed that they share similar 3D structures of the extracellular loops. Thus, the NMR structures of eLP1 and the N-terminal first part of eLP2 of TP shared a high degree of similarity with hEP3 (Figure 1B), and therefore they were directly adapted for the 3D model of the corresponding loops of hEP3 (Figure 1A). The 3D structure of the C-terminal region of eLP2 of EP3 was obtained by a fragment search⁴⁰. The NMR

structure of eLP3 of TP, which has only one residue less than eLP3 of hEP3, was also adapted for the 3D model of hEP3 eLP3. The homology sequence search was used to create the structural model of the second half of the hEP3. To complete the working model containing the ligand recognition and binding sites, the constructed structural models of the individual EP3 eLPs were connected to the corresponding TM domains using the approach described previously⁴¹. The assembled 3D working model after refinements (disulfide bond formation between eLP1 and eLP2, 1000 steps of energy minimization and dynamic study) are shown in Figure 1C.

3.2. Localization of the PGE₂ recognition pocket on the 3D model of the wild type hEP3

In addition to the TM ligand-deposited pocket, another PGE₂-binding pocket on the extracellular domain was visualized through the docking of PGE₂ with the constructed 3D working model of hEP3 (Figure 2A). The extracellular domain pocket formed by eLPs is a door-like opening between the extracellular environment and the TM domains. It is accessed by the initial binding of PGE₂ to the TM domain pocket of the receptor. Thus, we call it the “PGE₂ recognition pocket” in this study. The localized recognition pocket is similar to that of our earlier studies for human TP and IP, in which the non-conserved residues within the eLP2s are involved in the formation of specific ligand recognition pockets for TXA₂ and PGI₂. This was confirmed by site-directed mutagenesis and high resolution ¹H-NMR spectroscopic experiments^{39,41}. The localized PGE₂ recognition pocket on the 3D model also agrees with the recent finding that non-conserved S211 (Figure 2A) within the central region of eLP2 of hEP3 is involved in PGE₂ recognition. This was confirmed by site-directed mutagenesis and NMR spectroscopy⁴². A path for PGE₂, which moves from the recognition pocket into the TM pocket for the final signaling, was also observed (Figure 2B). This information support that the constructed 3D model can be used for further analysis of PGE₁ recognition.

3.3. Localization of the PGE₁ recognition pocket on the 3D model of the wild type hEP3

PGE₁ and PGE₂ are synthesized from similar omega-6 unsaturated fatty acids (UFAs), DGLA and AA, respectively. The structural difference is minimal. PGE₁ has one double bond and PGE₂ has an additional cis double bond. Regarding ligand recognition, there are three possible mechanisms. One possibility is that PGE₁ and PGE₂ bind to the same pocket on the same receptor. The second possibility is that the receptor has two separate ligand recognition sites. Another possibility is that both PGE₁ and PGE₂ bind to the same pocket with different configurations, thus contacting different key residues of the receptor. To test these hypotheses, PGE₁ and PGE₂ were docked with the 3D model of the constructed hEP3 to localize the recognition pocket. The results showed that PGE₁ fits into the same pocket as PGE₂, which is formed by three eLPs (Figure 3). The disulfide bond between eLP1 and eLP2 is involved in the formation of the pocket (Figure 3). This led us to predict that PGE₁ and PGE₂ share the same recognition pocket. This explains the mechanism of how PGE₁ could reduce the PGE₂ binding to EPs *in vitro* and *in vivo* through competitive binding. However, it could not address the fundamental question of how EP recognizes PGE₁ and PGE₂ differently. Through further analysis of the docking results, we found that the orientation of PGE₁ and PGE₂ in the recognition pocket of EP3 is significantly different (Figure 4). For example, with respect to the position of the identified key residue, S211, the carboxylic group of PGE₂ is facing S211, while the carboxylic group of PGE₁ is rotated 180 degrees away from S211. The distance between docked PGE₂ and S211 is approximately 3.6 Å, which suggests that they could form intermolecular contacts (intermolecular force, Figure 4B). In contrast, the distance between S211 and the docked PGE₁ increased to approximately 5.3 Å, which excludes the possibility of an intermolecular interaction between PGE₁ and S211 (Figure 4A). In other words, S211 is important in PGE₂ recognition, but has little to do with PGE₁ recognition. The different numbers of double bonds in PGE₁ and PGE₂ can affect their conformations, which was shown by Sybyl-X 2.0 (supplemental Figure 1). These results led us to propose a model demonstrating the significant difference between PGE₁ and PGE₂ docking in the EP recognition pocket.

3.4. Application of computational study in creating an EP3 mutant, which recognize PGE₁ and PGE₂ differently

Although many studies have been reported on the binding and signaling of the four EPs, little information regarding the molecular mechanisms of PGE₁ and PGE₂ binding to EPs is available. A specific PGE₁ agonist could be a promising drug, which relax smooth muscle by promoting blood circulation and compete with PGE₂ to reduce inflammation. Our computational study helped to identify the key residue involved in distinguishing the binding between PGE₁ and PGE₂ on the receptor. It also provided us with the requisite information needed to create a PGE₁-specific EP3 mutant by changing S211 to a different type of amino acid residue. After virtually replacing S211 with hydrophilic-charged residues, hydrophilic-neutral residues and hydrophobic residues, we found that S211L (one of previously reported mutants)⁴⁰ demonstrated that PGE₂ could not easily dock into the ligand recognition pocket (Figure 5B) and had lower binding affinity than the wild type hEP3 (Figure 5A). The replacing Ser to Leu residue by a point-mutation would not change TM domain binding domain. Unlike PGE₂, PGE₁ was able to bind to both hEP3 and S211L recognition pockets (Figure 6). The S211L receptor does not allow for PGE₂ to fit correctly into the binding pocket unlike the wild type hEP3. When S211 was changed to Leucine, PGE₂ could no longer form a hydrogen bond with the carboxylic acid of PGE₂ and the hydroxyl group of S211, thus reducing the binding affinity. It suggests that S211L could be a strong candidate for the preparation of PGE₁-specific EP3 ligands, which could block PGE₂ signaling while PGE₁ maintaining signaling.

3.5. Characterization of the hEP3 S211L mutant using highly sensitive and specific GPCR-mediated signaling assay in live cells

We have established the cell lines stably expressing the EP3 S211L and wild type in HEK293 cells, previously²⁰. The binding study showed that S211L mutant lost binding to ³H-PGE₂ (Figure 7). The K_d and B_{max} values were approximately 0.42 and 28.3 nM for the wild type EP3, respectively, but not quantifiable for the S211L mutant (Figure 7A). This indicated that S211 is a key residue involved in the

binding pocket for PGE₂ on the EP3. The proposed H-bond interaction between PGE₂ and S211 in the wild type EP3 is supported by the S211L mutant, which replaced the H-bond forming Serine with non-H-bond forming Leucine. It should be indicated that a similar assay to measure the binding of PGE₁ to the S211L mutant could not be performed, because isotope labeled-PGE₁ is not commercially available. However, the EP3 signaling assay could be used as an indirect measurement. Two major signaling activities, decreasing cAMP level and triggering calcium mobilization upon binding of the EP3 to PGE₁ or PGE₂ have been identified previously^{32,33}. Wild type hEP3 couples to the Gi protein, mediating the inhibition of cAMP upon activation of the receptor. To confirm hEP3-mediated cAMP inhibition, we have performed a cAMP assay. In this assay, EP2, which couples to Gs, was used as a positive control to show an increase in cAMP production by PGE₁ and PGE₂, and HEK293 was used as a negative control (Figure 7B). PGE₁ and PGE₂ both stimulated cAMP production in EP2 whereas hEP3 was not able to accumulate cAMP by PGE₁ or PGE₂.

The inhibition of cAMP formation through HEK293 expressing EP3 wild type cells (EP3wt) and EP3 S211L mutant cells (EP3S211L) was performed to determine PGE₁ and PGE₂. 1μM forskolin was used to stimulate adenylyl cyclase in the presence or absence of PGE₁ or PGE₂ in the cells. PGE₁ significantly and equally inhibited cAMP formation of both EP3wt and EP3S211L cells (Figure 8A). However, even though PGE₂ significantly inhibited cAMP formation of EP3wt by 3 nM and 30 nM, it failed to inhibit cAMP formation at the same degrees in EP3S211L cells (Figure 8B). Furthermore, using the stable cell line expressing EP2, which only couples to Gs to increasing cAMP level upon binding to PGE₁ and PGE₂ as a positive control, we found that EP3-mediated downregulation of cAMP was not large enough to be measured, as the basal level of cAMP is very low in HEK293 cells (Figure 7B). In contrast, the calcium mobilization assay is highly sensitive and can detect EP3 signaling mediated by PGE₁ and PGE₂ (Figure 8A). The developed calcium signaling assay has a number of additional benefits. The assay allows us to evaluate signaling activity in real-time in live cells, and also provides a method to monitor the signaling profile. These benefits are not available in the cAMP assay. This led us to establish a Ca²⁺ mobilization

assay in the EP3 live cells to monitor the wild type and S211L mutant activities at sensitive and multi-dimensional mode (Figure 9A). The cells expressing the wild type EP3 responded to the simulations of both PGE₁ and PGE₂ equally (Figure 9A left). In contrast, the untransfected HEK293 cells do not exhibit signaling activity (Figure 9A right). S211L mutant lost PGE₂ binding and calcium signaling activity for 100 nM of PGE₂-stimulation (Figure 9B, closed bars), but retained high signaling activity for an equal concentration of PGE₁ (Figure 9B, open bars). We have previously reported that S211L and R214 residues are important in PGE₂ recognition, but not N217 within the surrounding residues of the S211 site in the second extracellular loop of human EP3 by NMR spectroscopy and mutagenesis⁴². The cells expressing wild type EP3 and the internal control, R214L showed high signaling in response to both PGE₁ and PGE₂ (Figure 9B). Furthermore, a dose-dependent signaling assay was performed to confirm the selective PGE₁ recognition of S211L mutant. The S211L mutant mediated PGE₁ calcium signaling in a concentration-dependent manner (Figure 10A column 1 and Figure 10B). In contrast, the mutant lost the response to PGE₂ using the same concentrations (Figure 10A column 2 and Figure 10B). The untransfected HEK293 cells were used as a negative control (Figure 10 columns 3 and 4). PGE₁ or PGE₂ treated HEK293 cells could not be stimulated by any concentrations from 1 to 100 nM (data not shown). To further confirm the loss of PGE₂ binding of the S211L mutant, EP3wt and EP3S211L mutant cells were first treated with PGE₂, followed by PGE₁. The EP3wt was stimulated by both treatments (Figure 11A), whereas the EP3S211L mutant was only stimulated by PGE₁ treatment (Figure 11B), further supporting the result that PGE₂ lost binding to EP3S211L mutant, but is retaining PGE₁ binding and signaling. These results provide evidence supporting the prediction of the computational study; S211 is one of the key residues involved in the specific recognition for PGE₂, but has no impact on PGE₁. It also supports the hypothesis that PGE₂ and PGE₁ dock into the same ligand recognition site of hEP3 with differing configurations, allowing for contact with different key residues of the receptor.

4. DISCUSSION

Thus far, the structural and functional relationship of ligand selectivity in binding to prostanoid receptors, which include four PGE₂ receptors (EP1-4), TXA₂ receptor (TP), PGD₂ receptor (DP) and PGF₂ receptor (FP), are poorly characterized. One of the major obstacles in prostanoid receptor research is the lack of X-ray structures to reveal specific ligand binding sites. This study provides an initial step in understanding the ligand-specific recognition of prostanoids at their receptors using integrative approaches before X-ray structures are available. All of the prostanoid receptors contain a highly conserved TM domains²⁵, where they form ligand-deposited pockets similar to other GPCRs bound to small molecules⁴⁴. However, the conserved TM residues of the prostanoid receptors have little impact on their ligand selectivities. Thus, for decades, many studies focusing on the TM ligand-deposited site could not clearly identify the key residues involved in ligand selectivity, especially for the prostanoid receptors, EP1-4, which bind to PGE₁ and PGE₂ primarily to mediate diverse biological functions. This study aims to elucidate the relationship between structure and selectivity of EP3 at the initial step before the ligand is deposited into the TM binding pocket. Our earlier study using TP^{34-37,54-60} and IP⁴⁵ as models revealed that the residues within the non-conserved extracellular loops, especially within eLP2 are involved in forming initial recognition sites for TXA₂ and PGI₂ before they are deposited into the TM pockets^{34,37,39,45,54-57,60}. This finding has provided a basis to further characterize the molecular mechanisms of ligand selectivity, demonstrating how one PGE receptor mediates biological functions thorough different recognition of two nearly identical ligands, PGE₁ and PGE₂. Utilizing both the X-ray structure of β_2 -adrenergic receptor as TM domain templates and NMR structure of TP as templates for the extracellular loops, a 3D-working model of hEP3 was constructed (Figure 1-2). There are EP3 isomers which have differences in their C-terminus sequences from residue 360-390, existing in the cytoplasmic region. These sequence differences would not affect ligand binding as the ligand recognition site exists in the extracellular region. It indicated that using similar approaches, 3D-working models of other EPs could also be constructed in order to localize their specific ligand-recognition pockets for PGE₁ vs PGE₂ on the extracellular domains. When PGE₁ and PGE₂ were docked with the working model, it revealed that only

one pocket on the extracellular loops fit both ligands. However, they adapt different configurations with respect to their interaction with S211 in the pocket. The specific recognition of PGE₂ depends on S211 contact, but PGE₁ is independent of S211. The structural finding was validated by site-directed mutagenesis using S211L mutant. The [³H]-PGE₂ binding assay has directly shown that the S211L mutant lost its PGE₂ binding. This was also supported by the experiment shown in Figure 11, in which PGE₂ did not prevent the S211L mutant activation. This experiment indicates that PGE₂ lost its ability to bind to the EP3 S211L mutant, and turned down calcium signaling, but retained PGE₁ mediated calcium binding. For the first time, we demonstrated that one residue can control ligand selectivity in a prostanoid receptor.

As described previously, PGE₂ mediates inflammation and cancer, but PGE₁ can protect against vascular damage as well as GI insult from NSAIDs. Interestingly, PGE₁ was found to activate IP receptor at $pKi = 6.8^{25}$ as in EP receptors at 6.8 (hEP1)⁶¹, 8.0 (mEP2)⁶², 9.0 (mEP3)⁶², and 9.5 (hEP4)⁶³, which could be contributing to anti-inflammatory effects. Another possibility is the signaling differences of the EP3 receptor isoforms. These isoforms were reported to have different degrees, signaling or combination of signaling by the receptor activation^{64–66}. For example, EP3A and EP3B can inhibit forskolin-stimulated cAMP as well as produce calcium signaling. EP3C has less effect in inhibiting forskolin-stimulated cAMP, but produce calcium signaling. Some of the isoforms do not have calcium signaling, which could be involved in different effects observed between PGE₂ and PGE₁. Nevertheless, one of the approaches to reduce the side effects of these drugs is to develop specific PGE₁ agonists. Our modified residue, S211L, specifically mediates PGE₁ signaling and turns down the PGE₂ signaling, and could thus be used as a novel target in screening specific PGE₁ agonists. The S211L mutant could be used to test affinities, cAMP activity and calcium signaling properties of the current PGE₁ analogues (such as, Misoprostol and 11-deoxy PGE₁) to see if the receptor exhibits differential activation by the ligands with a double bond vs. single bond.

S211 was identified as an important residue for PGE₂ recognition, but not for PGE₁ recognition. It is possible that the identical or similar residues in the other three EPs and other prostanoid receptors

could function in a similar manner, turning-down PGE₂ signaling and retaining PGE₁ signaling. Through looking for the polymorphism and genetic mutation around S211, we may be able to further characterize the residues involved in the differential recognition of PGE₂ and PGE₁. It could explain the unknown pathophysiological mechanisms of why some individuals exhibit less chance of or even resistance to developing PGE₂-mediated inflammation.

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FIGURES LEGENDS

Figure 1. A) A homology model of the hEP3 transmembrane domains (TM, green color) using human β_2 -adrenergic receptor as a template and the extracellular loops (pink color) using NMR structures of eLPs of the human TP (hTP). B) Alignment of the eLPs between hTP and hEP3. C) Constructed 3D model of hEP3 by assembling the 3D models of the three extracellular loops (eLPs, pink color) connected to the models of the seven TM domains (green color) adapted from Figure 1A.

Figure 2. A) Localized PGE₂-recognition pocket for hEP3. S211 in the EP3 eLP2 is indicated. B) The PGE₂ binding pocket within the TM domains is shown.

Figure 3. Superimposed configurations of PGE₁ (Red) and PGE₂ (Blue) docked into the ligand recognition site of hEP3. The involved eLPs and the disulfide bond between eLP1 and eLP2 are indicated.

Figure 4. Docking of PGE₁ (A) and PGE₂ (B) with the wild type (WT) EP3 model. The relationships between the docked PGE₁/PGE₂ and S211 are shown. The distances between atoms which could form a hydrogen bond within PGE₁/PGE₂ and S211 are indicated.

Figure 5. Docking site of PGE₂ with the recognition pocket of the constructed (A) hEP3 model, and (B) S211L mutant model. The surface of the docking sites is displayed, and colored by cavity depth.

Figure 6. Docking site of PGE₁ with the recognition pocket of the constructed (A) hEP3 model, and (B) S211L mutant model. The surface of the docking sites is displayed, and colored by cavity depth.

Figure 7. A) Dose dependent curves for the binding of [³H]PGE₂ to HEK293 cells expressing wild type (squares) and S211L mutant (circles). B) PGE₁ and PGE₂-mediated cAMP signaling in the wild type

hEP3 cells. hEP2 and the untransfected HEK293 cells were used as positive and negative controls, respectively. 100 nM of PGE₁ and PGE₂ were used (n=3).

Figure 8. The inhibition of cAMP formation through HEK293 expressing EP3 wild type cells (EP3wt) and EP3 S211L mutant cells (EP3S211L). 1 μ M forskolin was used to stimulate adenylyl cyclase with/without PGE₁ (A) or PGE₂ (B) in the cells. * indicates statistical significance ($p<0.05$) between EP3wt “0” vs. “3” or “30”. “†” indicates statistical significance ($p<0.05$) EP3S211L “0” vs. “3” or “30”. “a” indicates statistical significance ($p<0.05$) between EP3wt and EP3S211L within the same concentration.

Figure 9. A) PGE₁ and PGE₂ mediated-calcium signaling for live cells. The real-time live cell images and plots of the calcium mobilization signaling for the wild type hEP3 cells (left panels) and the untransfected HEK293 cells (right panels) induced by PGE₁ or PGE₂ were shown. The light intensity produced by pre-loaded Fluo-8 AM bound to intracellular calcium were used to calculate the strength of the calcium signaling in live cells (n=3). B) Comparisons of the differences between PGE₁ (open bars) and PGE₂ (closed bars)-induced calcium signaling on the HEK293 cells expressing S211L, R214L and the wild type (WT). The real time calcium assay was performed by treating the cells with 100 nM of PGE₁ or PGE₂ using Fluo-8 AM as an intracellular calcium indicator, n=3.

Figure 10. A) Comparisons of calcium signaling assays on the HEK293 cells expressing EP3S211L mutant induced by 100 nM PGE₁ (column 1) and 100nM PGE₂ (column 2). The untransfected HEK293 cells (column 3 and 4) were used as controls. B) Dose response curve of the HEK293 cells expressing EP3S211L mutant induced by PGE₁ and PGE₂. The plots were obtained by subtracting the maximum intensity from the base line intensity (n = 3).

Figure 11. Comparisons of a successive Ca^{2+} signaling response in EP3wt and EP3S211L mutant by PGE_2 followed by PGE_1 . The cells were first stimulated by PGE_2 (1 μM). After 25 seconds, the cells were stimulated by PGE_1 (1 μM). The experiments were continuously recorded under a Nikon Ti-S eclipse microscope using NIS Elements 2.35 software.

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Figure 1

Figure 2

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Figure 4

Figure 5

Figure 6

Figure 7

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B

Figure 8

Figure 9

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Figure 10

Figure 11