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Characterization of the prostaglandin H_2 mimic: Binding to the purified human thromboxane A_2 receptor in solution ☆

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ABSTRACT

For decades, the binding of prostaglandin H_2 (PGH_2) to multiple target proteins of unrelated protein structures which mediate diverse biological functions has remained a real mystery in the field of eicosanoid biology. Here, we report that the structure of a PGH_2 mimic, U46619, bound to the purified human TP, was determined and compared with that of its conformation bound to the COX-downstream synthases, prostacyclin synthase (PGIS) and thromboxane A_2 synthase (TXAS). Active human TP protein, glycosylated and in full length, was expressed in Sf-9 cells using a baculovirus (BV) expression system and then purified to near homogeneity. The binding of U46619 to the purified receptor in a nonionic detergent-mimicked lipid environment was characterized by high-resolution NMR spectroscopy. The conformational change of U46619, upon binding to the active TP, was evidenced by the significant perturbation of the chemical shifts of its protons at H3 and H4 in a concentration-dependent manner. The detailed conformational changes and 3D structure of U46619 from the free form to the TP-bound form were further solved by 2D ^{1}H NMR experiments using a transferred NOE (trNOE) technique. The distances between the protons of H11 and H18, H11 and H19, H15 and H18, and H15 and H19 in U46619 were shorter following their binding to the TP in solution, down to within 5 Å, which were different than that of the U46619 bound to PGIS and U44069 (another PGH_2 mimic) bound to TXAS. These shorter distances led to further separation of the U46619 α and ω chains, forming a unique "rectangular" shape. This enabled the molecule to fit into the ligand-binding site pocket of a TP model, in which homology modeling was used for the transmembrane (TM) domain, and NMR structures were used for the extramembrane loops. The proton perturbations and 3D conformations in the TP-bound U46619 were different with that of the PGH_2 mimics bound to PGIS and TXAS. The studies indicated that PGH_2 can adopt multiple conformations in solution to satisfy the specific and unique shapes to fit the different binding pockets in the TP receptor and COX-downstream enzymes. The results also provided sufficient information for speculating the molecular basis of how PGH_2 binds to multiple target proteins even though unrelated in their protein sequences.

Prostaglandin H_2 (PGH_2)[1] is an endogenous lipid molecule produced by cyclooxygenase from arachidonic acid (AA). The unstable PGH_2 is a potent ligand that activates the thromboxane A_2 (TXA_2) receptor (TP) and therefore mediates the receptor signaling that causes thrombosis and vasoconstriction, which can lead to strokes and heart attacks [1–2]. On the other hand, PGH_2 also serves as a common substrate and can be isomerized to the biologically active prostaglandins (PG) E_2 (PGE_2), PGD_2, PGF_2, PGI_2, and thromboxane A_2 (TXA_2) by their corresponding COX-downstream synthases, PGE_2 synthase (PGES), PGD_2 synthase (PGDS), PGF_2 synthase (PGFS), PGI_2 synthase (PGIS), and TXA_2 synthase (TXAS) (Fig. 1, [1–10]). The synthesized prostanoids mediate diverse and opposite pathophysiological processes within the vascular [1–2,11–14], nervous [15], and reproductive [16–18] systems as well as cancers [19–20]. The primary structures of the TP receptor and the COX-downstream synthases are unrelated. The TP is a G protein-coupling receptor located on the cell membrane with seven transmembrane domains, while the COX-downstream synthases belong to P450 and glutathione-related proteins and other enzymes [15].

The molecular mechanisms of the PGH_2 and its multiple protein-binding properties have been addressed for decades. However, very little information had been made available until

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[1] *Abbreviations used:* AA, arachidonic acid; TXA_2, thromboxane A_2; TXAS, TXA_2 synthase; PGI_2, prostacyclin or prostaglandin I_2; PGIS, PGI_2 or prostacyclin synthase; PGH_2, prostaglandin H_2; TP, TXA_2 receptor; ER, endoplasmic reticulum; NMR, nuclear magnetic resonance; DQF-COSY, double-quantum-filtered correlation spectroscopy; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total correlation spectroscopy; 1D, one-dimensional; 2D, two-dimensional; 3D, three-dimensional; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

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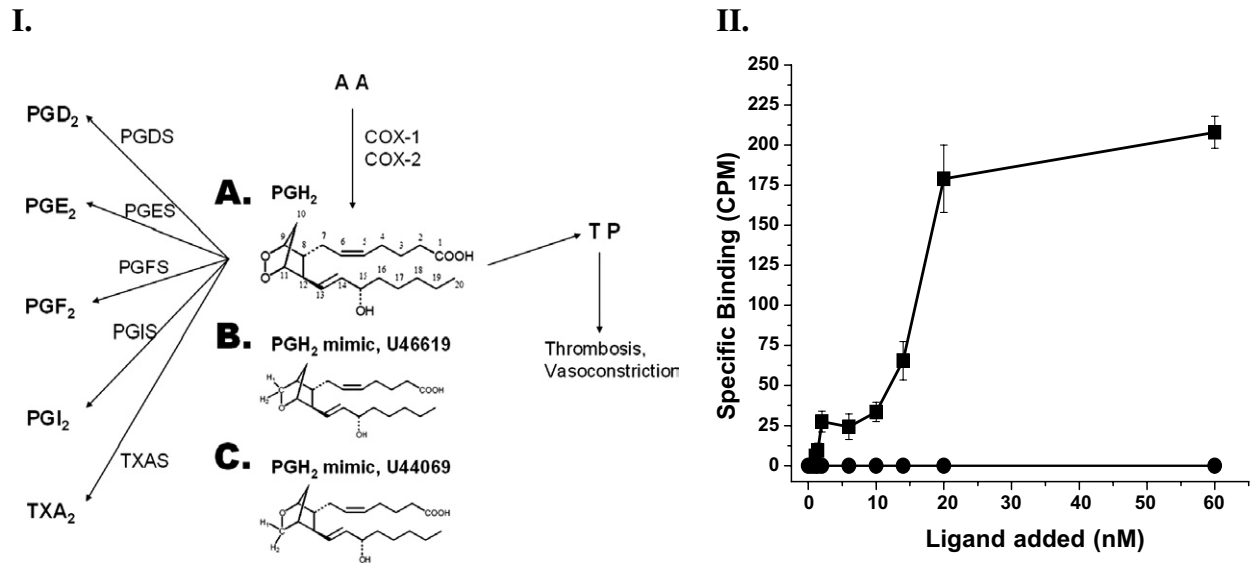

Fig. 1. Panel I: The multiple target proteins of PGH_2 synthesized from arachidonic acid (AA) through the COX pathway. The COX-1 and COX-2 enzymes convert AA to PGH_2 (A), which serves as a common substrate for COX-downstream synthases including PGDS, PGES, PGFS, PGIS, and TXAS, and also serves as an agonist for the TP. The chemical structures of the PGH_2 mimics, U46619 (B), and U44069 (C) are also shown. Panel II: The specific ligand binding activity of the purified TP from Sf9 cells. The purified TP protein (■) in PBS was incubated with increasing amounts (0–60 nM) of the ligand, [^{3}H]U46619. Unlabeled U46619 (1 μM) was added to the Sf9 cells as a negative control (●) before the radio-labeled ligand was added. The results are representative data from three assays (n = 3) and are shown as means ± the standard error.

the very recent successful purification of a large amount of recombinant TXAS [21] and PGIS [22]. Characterization of the PGH_2 mimics' interaction with the purified PGIS and TXAS in solution, using high-resolution NMR spectroscopic techniques, has revealed that the PGH_2 mimics' (U44069 and U46619) structures are highly flexible in solution. The PGH_2 mimics underwent different conformational changes when bound to the active and purified TXAS and PGIS proteins in solution [21–22]. The altered conformations were favorable for the docking of PGH_2 into the pockets of the enzymes' substrate-binding sites with different configurations. For example, the free form of PGH_2 in solution adopts a relaxed conformation with wide open α and ω chains. However, the conformation changes to a compact, oval shape with shorter distances between the chains when bound to PGIS and TXAS [21–22]. These observations have led us to hypothesize that PGH_2 could adopt multiple conformations to specifically recognize the different target proteins. The PGH_2 conformation that was bound to the TP-ligand pocket mediates signaling, which is different and specific in comparison to its binding with other COX-downstream synthases.

However, there is minimal structural information available for the molecular basis of PGH_2 bound to TP. One of the major obstacles is the lack of an approach for preparing a large amount of the purified TP protein for structural characterization of the interaction between the receptor and its ligand. Recently, we reported the characterization of a solution structure of a TP ligand interacting with a constrained peptide that mimics the receptor–ligand recognition site by high-resolution NMR spectroscopy using the trNOE technique. This revealed useful information for understanding the molecular mechanism of the ligand–receptor interaction. In this paper, an attempt has been made to obtain purified TP protein and allow it to interact with PGH_2. The conformation of PGH_2, as a ligand bound to TP, was characterized by the binding of a PGH_2 mimic, U46619, to the purified TP using a previously established trNOE technique. The detailed solution structure of U46619 bound to TP was solved, then utilized to reveal the specific conformation, and compared with the other PGH_2 mimics bound to PGIS and TXAS. Thus, the conformation-dependent activation of the different target proteins by PGH_2 was demonstrated.

Experimental procedures

Materials

^2H_2O, ethanol-d_6 and DSS, (sodium 2, 2-dimethyl-2-silapentane-5-sulfonate) were purchased from Cambridge Isotope Laboratories (Andover, MA). U46619, a stable analog of PGH_2, was obtained from Cayman Chemicals (Ann Arbor, MI). The detergent, *n*-dodecyl-β-D-Maltoside (DM) was purchased from Calbiochem (San Diego, CA). All other chemicals were purchased from Sigma (St. Louis, MO).

Expression and purification of the human TP

The expression and purification of the recombinant human TP protein are described as follows. Briefly, human TP cDNA was subcloned into the pVL1392 vector, which is suitable for the Baculovirus (BV) expression. A 6His-tag DNA sequence was also added to the isolated TP cDNA at the C-terminal position of the receptor protein to generate the pVL1392-TP-6His construct using a PCR approach. The pVL1392-TP-6His was packed into BV with Orbigen Sapphire DNA and the recombinant BV was then prepared and used for overexpressing the full length TP protein in Sf-9 cells. The TP protein was extracted from the Sf-9 cells using 1% DM, separated twice by ultracentrifugation for 1 h at 4 °C and 250,000*g*, and purified by Fast Protein Liquid Chromatography (FPLC) on a Superdex-75 column (1 × 45 cm) with a flow rate of 0.2 mL/min using PBS with 0.2 mM DM. The fractions containing the active TP protein were identified by binding assays, polyacrylamide gel electrophoresis (PAGE) analysis, and Western blot analysis.

Electrophoresis, Western blot analysis, and ligand binding assay

The purified TP receptor was separated by 10% PAGE under denaturing conditions and then stained by protein staining or transferred to a nitrocellulose membrane for Western blot analysis, in which a band recognized by anti-human TP receptor antibody (Cayman Chemicals) was visualized by a second antibody linked to horseradish peroxidase, as previously described [23]. The