Expression and purification of human mPGES-1 in E. coli and identification of inhibitory compounds from a drug-library

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INTRODUCTION

Synthesized from membrane-derived arachidonic acid via the reactions of cyclooxygenases and prostanoid synthases, prostaglandins have multiple roles in human physiological processes (1, 2). Arachidonic acid is converted by cyclooxygenase-1 (COX-1) or cyclooxygenase-2 (COX-2) to PGH2 and then metabolized by specific prostanoid synthases to PGE2, PGD2, PGF2, or thromboxanes. In particular, prostaglandin E synthases (PGESs) convert PGH2 to PGE2, which regulates multiple physiological processes including inflammation (3-5), reproduction (6), or tumorigenesis (7, 8). Proinflammatory stimuli induce the production of PGE2 in macrophages and other tissues (3, 4, 9-12) and elevated levels of PGE2 have been shown to mediate fever (13) and pain (14).

Three kinds of human PGE synthases have been identified: a cytosolic prostaglandin E2 synthase (cPGES), which consists of 160 amino acids (15), and two microsomal membrane-associated prostaglandin E2 synthases (mPGES-1 and mPGES-2) with 152 (9) and 373 amino acid residues (16), respectively. Two of these synthases, cPGES and mPGES-2, are constitutively expressed and promote immediate PGE2 production via constitutive COX-1 as part of cellular homeostatic maintenance (15). In contrast, mPGES-1 and COX-2 expression can be induced by proinflammatory stimuli in various tissues (3, 4, 10). Traditional nonsteroid anti-inflammatory drugs (NSAID) or specific COX-2 inhibitors lower the level of PGE2 and inhibit the synthesis of PGL2, thromboxane A, or PGD2 (17). Hence, the selective inhibition of mPGES-1 would generate anti-inflammatory effects without unwanted side effects involving homeostasis. Gene knockout experiments with mice lacking mPGES-1 showed impaired inflammatory and pain responses (18), implying that mPGES-1 may be a promising drug target against chronic inflammatory diseases such as rheumatism (19).

Recently, a few compounds capable of inhibiting the catalytic activity of mPGES-1 have been reported. A stable PGH2 analog (20) and NS-398 (21) were shown to inhibit mPGES-1 with a marginal potency (IC50 = 10-20 μM). Structure-activity relationship (SAR) studies using MK-886, an inhibitor of 5-lipoxygenase activating protein (FLAP), led to the generation of highly potent mPGES-1 inhibitors having an indole carboxylic acid structure (22), but they displayed low potency and selectivity in cell-based experiments, indicating that further improvement and experimentation is required (22). High throughput screening of these inhibitors allowed the identification of novel mPGES-1 inhibitory compounds, and optimization of one selected compound identified a highly potent phenanthrene imidazole derivative (M63) with low IC50 value (μM range) and high bioavailability (23).

Although the development of novel inhibitors of mPGES-1 has received great attention, the variety of inhibitors with different chemical backbone structures and mPGES-1 structure information remain limited although the structure of mPGES-1 has been modeled and the substrate binding sites characterized (24). A higher resolution structure of mPGES-1 is required to facilitate the design of novel inhibitors and the optimization of previously identified inhibitors. In this report, the expression of recombinant mPGES-1 in E. coli has been optimized, the expressed protein purified, and its oligomeric state characterized. Additionally, a commercial library consisting of bioactive and drug compounds was screened for inhibitory compounds with new structural scaffolds.
RESULTS AND DISCUSSION

Optimization of mPGES-1 expression in E. coli

The expression of membrane-associated proteins in heterologous hosts has had limited success, as in the case of COX-1 (25) or cytochrome P450 (26). In many cases, overexpression of membrane proteins was found to decrease growth rates or induce cell-death in the expression host. Expression of recombinant human mPGES-1 in insect cells or E. coli has been reported, expressed in the latter at a level of 0.2-1 mg per L of culture (27) in the membrane fraction; and in a baculovirus system, the expression levels were similar to E. coli expression systems (28).

There are several factors that affect the level of recombinant protein expression in E. coli other than the strength of the promoters. The frequency of codons that are rarely used in the target sequence inserted into E. coli is one of the critical factors that determine the expression level. Several rare codons in the coding sequence of human mPGES-1 are suppressed in the Rosetta strain, but it has three CGG codons that are not suppressed by the Rosetta strain and are rarely used in E. coli. To avoid the potential retardation of mPGES-1 translation, all three CGG codons were changed to CGC codons, highly recognized by the tRNAs in E. coli, creating a mutant mPGES-1 with three silent mutations. When the wild-type and mutant sequences were expressed in various E. coli strains, the Rosetta (DE3) strain had a 5-7-fold greater expression relative to the BL21(DE3) strain. In addition, the codon-frequency mutant displayed an approximate 2-3-fold increase in expression relative to the wild-type (Fig. 1A, B). These results indicated that the presence of rare codons in human mPGES-1 hindered high-level expression in E. coli, and the introduction of E. coli-friendly codons and the use of E. coli strains supplemented with tRNAs for rare codons effectively increased the expression level of these proteins.

Purification and characterization of mPGES-1

The majority of the expressed mPGES-1 was recovered in the membrane fraction, completely dissolved in 4% Triton X-100 solution, and successfully purified to homogeneity using Ni-NTA and ion exchange columns (Fig. 1C). A purified 18.8 kDa band, detected with anti-His tag antibody, was observed after ion exchange chromatography with a SP-column and matched the calculated size (19 kDa) of the recombinant His-tag labeled mPGES-1. The final yield of the purified protein was 0.5 mg per L culture.

The enzymatic properties of the purified mPGES-1 were characterized by measurement of the reaction rate of the purified mPGES-1 at different concentrations of PGH2 using a competition assay with PGE2-labeled alkaline phosphatase to PGE2-specific antibody (Fig. 2). The $K_m$ and $V_{max}$ values, calculated from Lineweaver-Burk plot as $2.4 \pm 0.25 \mu M$ and $3.5 \pm 0.08 \text{nmol.s}^{-1}$, respectively, were in the same range as the mPGES-1 obtained from insect cells (28).

Characterization of oligomeric state of mPGES-1

The apparent size of mPGES-1-Triton X-100 complex was previously measured as 215 kDa in hydrodynamic studies and determined to be a trimer in the protein-detergent complex (27). Thus, to chemically cross-link the purified His-tag labeled mPGES-1 and determine its oligomeric state, the enzyme was incubated with glutaraldehyde and analyzed by SDS-PAGE, yielding protein bands at 38 and 57 kDa detected by anti-His tag antibody and corresponding to dimeric and trimeric forms of mPGES-1 (Fig. 3A). When lysosome, a monomeric protein, was treated with the same concentration of glutaraldehyde, only the monomer band was detected (Fig. 3B), indicating that the high-molecular weight bands observed after cross-linking mPGES-1 represent true oligomers rather than aggregates.

Fig. 1. Overexpression and purification of mPGES-1. (A) Expression of weight and codon frequency of mutant mPGES-1 in E. coli as determined by 18% SDS-PAGE. (B) Western blot using anti-His tag antibodies: lane M, molecular weight markers; lane 1, crude extract of BL21(DE3) expressing wt-mPGES-1; lane 2, crude extract of Rosetta (DE3) expressing wt-mPGES-1; lane 3, crude extract of BL21(DE3) expressing codon frequency mutant mPGES-1; and lane 4, crude extract of Rosetta (DE3) expressing codon frequency mutant mPGES-1, ~20 μg of proteins loaded per lane. (C) Purification of mPGES-1. Fractions from each purification step analyzed by 18% SDS-PAGE (upper panel) and western blot (lower panel): lane M, molecular weight markers; lane 1, crude extract of Rosetta (DE3) cells; lane 2, crude extract (about 20 μg of protein) Rosetta (DE3) expressing codon frequency mutant mPGES-1; lane 3, membrane fraction from cell lysate; lane 4, the purified mPGES-1 after Ni-NTA column (about 0.5 μg); lane 5, the purified mPGES-1 (about 0.5 μg) after Q-sepharose column. Arrows indicate the expressed mPGES-1.

Fig. 2. Enzymatic properties of the purified recombinant mPGES-1. The activity of the mPGES-1 measured in various PGH2 concentrations.
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Identification and characterization of novel mPGES-1 inhibitors
The initial screening of a chemical library of 1,040 bioactive or drug compounds to identify novel mPGES-1 inhibitors yielded oxacillin and dyphylline as having inhibitory activity. Further analysis showed that these two compounds inhibited mPGES-1 within the concentration range of 100-200 μM, and IC₅₀ values of oxacillin (Fig. 4A) and dyphylline (Fig. 4B) were calculated as 0.11 mM and 0.23 mM, respectively. The inhibition mechanisms of dyphylline and oxacillin were examined by measuring $K_m$ and $V_{max}$ values from Eadie-Hofstee plots of mPGES-1 activity in the presence of these compounds. The $K_m$ values of mPGES-1 in the presence of dyphylline or oxacillin increased, whereas the $V_{max}$ values were unchanged in their Eadie-Hofstee plots (Fig. 4C and 4D indicating that these compounds were competitive inhibitors and suggested that both oxacillin and dyphylline were bound close to the PGH₂ binding pocket of mPGES-1. Oxacillin is an antibiotic that contains a β-lactam ring structure and dyphylline is bronchodilator agent that contains a methylxanthine structure, neither of which structural moieties have been previously reported as inhibitors of mPGES-1.

Optimization of oxacillin or dyphylline, however, is required for developing highly potent mPGES-1 inhibitors.

In summary, an optimized expression of human mPGES-1 in E. coli was achieved and the purified enzyme shown to be primarily in the trimeric state and to possess enzymatic characteristics consistent with previously reported parameters. The mPGES-1 product was then used to screen for inhibitors with novel structures from a commercial chemical library of bioactive compounds, identifying two compounds, oxacillin and dyphylline, for the first time as competitive inhibitors of mPGES-1. Although their IC₅₀ values were relatively higher than known inhibitors, the novel inhibitory activity of oxacillin and dyphylline against mPGES-1 may provide valuable insight for the design of more, potent mPGES-1 inhibitors.

MATERIALS AND METHODS

Materials
Escherichia coli strain DH5α was used for the amplification of
the expression vector and E. coli Rosetta (DE3) (Novagen, USA) used for the expression of mPGES-1. Luria Broth medium and agar (Merck, Germany) was used for the E. coli growth media, all restriction enzymes used for DNA digestion obtained from New England Biolabs (USA), and isopropyl-β-D-thiogalactopyranoside (IPTG) obtained from Bio Basic, Inc., Canada. The mouse anti-His-tag antibody, horseradish peroxidase (HRP) labeled anti-mouse antibody, stable peroxidase substrate buffer, and polyvinylidene fluoride (PVDF) membrane for western blotting were purchased from Santa Cruz, Sweden, respectively. Prostaglandin H2 (PGH2), oxacillin, and streptomycin, all restriction enzymes used for DNA digestion originated from MicroSource, USA.

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ature with moderate shaking, and finally washed with wash buffer (Assay Designs Kit). The color developing reaction was initiated by adding 200 μl of p-nitrophenyl phosphate (pNPP) substrate solution for 4 h at room temperature, terminated by adding 50 μl of stop solution, and the absorbance of the solution at 405 nm measured using a plate reader (DYNEX, USA). Inhibitory activity by test chemical compounds was measured by the incubation of 2 μl of a compound in dimethyl sulfoxide (DMSO) with mPGES-1 for 30 min prior to the addition of PGH₂. Protein concentrations were measured by the Lowry method (30) using bovine serum albumin as a standard.

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REFERENCES


