A role of prostaglandin transporter in regulating PGE₂ release from human bronchial epithelial BEAS-2B cells in response to LPS

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Abstract

Naturally occurring prostaglandin E₂ (PGE₂) plays a role in inflammatory responses through eicosanoid signaling pathways. PGE₂ is impermeable to cell membranes at physiological pH and needs solute carrier across the membranes; however, it remains unclear how intercellular concentrations of PGE₂ are regulated under the condition of inflammation. We aimed to clarify a role of organic anion-transporting polypeptide 2A1 (OATP2A1/*SLCO2A1*), also known as prostaglandin transporter (PGT), in PGE₂ release from cells. Human bronchial epithelial BEAS-2B cells were treated with lipopolysaccharide (LPS), and PGT inhibitors were tested to evaluate contribution of PGT to PGE₂ release by assessing its extracellular concentration and characterizing PGT-mediated PGE₂ efflux in *Xenopus laevis* oocytes. As a result, LPS elevated mRNA expression of a pro-inflammatory cytokine IL6 and extracellular concentration of PGE₂ in human bronchial epithelial BEAS-2B cells. PGT inhibitors tested (e.g. bromocresol green (BCG), bromosulfophthalein (BSP), and PGB₁) significantly inhibited efflux of PGE₂ from oocytes expressing PGT. Similarly, the amount of released PGE₂ from the BEAS-2B cells decreased in the presence of BCG and BSP by 45 and 44% respectively while TGBz increased the concentration by 71%, suggesting that PGT mediates the release. In conclusion, these results imply a role of PGT in regulating intra- and extracellular concentrations of PGE₂ in response to cells under inflammatory conditions.

Key Words

- ▶ prostaglandins
- ▶ cytokines
- ▶ cell culture
- ▶ PGT

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Introduction

Prostaglandin E₂ (PGE₂) plays a major role in eicosanoid signaling pathways, which are well preserved among vertebrates. Naturally occurring PGE₂ exerts diverse physiological actions such as inflammatory responses, uterine contraction, bone resorption, and induction of hyperthermia (Smith 1992). PGE₂ is synthesized via cyclooxygenase (COX) 1 and 2 from arachidonic acid

(Helliwell *et al.* 2004), and the precursor is further converted to PGE₂ by cytosolic or microsomal PGE₂ synthase (c/m PGES). PGE₂ signals in either autocrine or paracrine manners through G-protein-coupled PGE receptors (e.g. EP₁, EP₂, EP₃, and EP₄), which differ in tissue distribution; therefore, PGE₂ activates different G proteins depending on the subtype of receptor that is bound to and

then transduces diverse physiological actions (Bos *et al.* 2004). The downstream events have been studied extensively; however, current evidence is still missing the fundamental basis of how release of PGE₂ is regulated.

Prostaglandins (PGs) including PGE₂ are organic anions with pKa values of around 5 and exist in charged form at physiological pH (Roseman & Yalkowsky 1973). Hence, PGs are impermeable to cell membranes (Bito & Barood 1975) and have been suggested not to cross plasma membranes without an aid of transporter systems. There is substantial evidence that organic anion-transporting polypeptides (OATPs) are involved in translocation of PGE₂ across the plasma membranes (Abe *et al.* 1999, Tamai *et al.* 2000, Kullak-Ublick *et al.* 2001). Among those, OATP2A1/SLCO2A1 has been characterized as a solute carrier for PG transporter (PGT), particularly PGE₂, as it was isolated from adult human kidneys (Lu *et al.* 1996). PGT mediates PGE₂ cellular uptake, which can be driven by an exchange of organic anions such as lactate (Chan *et al.* 2002). PGT-mediated PGE₂ uptake from the extracellular environment is an initial step for degradation of PGE₂ by 15-hydroxy PG dehydrogenase (PGDH) in the cytoplasm (Nomura *et al.* 2004). Expression of PGT may be coordinately regulated with COXs (Bao *et al.* 2002). Furthermore, previous reports have shown that inflammatory effect of stannous chloride, by which heme oxygenase 1 is induced, is attenuated by a resultant elevation of PGE₂ cellular absorption mediated by augmented activity of PGT. Therefore, PGT expression may be associated with inflammatory responses.

On the other hand, several groups have implied that PGT is involved in release of PGE₂ from cells (Chan *et al.* 1998, 2002, Banu *et al.* 2003); however, contribution of PGT to PGE₂ release from cells remains elusive under physiological conditions. Therefore, we hypothesize that PGT secretes cytoplasmic PGE₂ from cells in response to extracellular inflammatory stimuli, which may accelerate PGE₂-mediated signaling or enhance inflammatory events in an autocrine manner. Previously, COX and EP receptors have been studied extensively as inflammatory regulators and established as pharmacological targets to control inflammatory reactions. EP receptor antagonists are currently in clinical trials as anti-inflammatory drugs. Compared with those two major factors, limited information is available regarding trans-cellular transport of PGE₂ by PGT. In this study, in order to understand the role of PGT in regulating extracellular concentrations of PGE₂ in response to inflammation, we studied the effect of PGT inhibitors on PGE₂ release and pro-inflammatory cytokine IL6 mRNA expression in

human bronchial epithelial cells treated with an inducing agent, lipopolysaccharide (LPS).

Materials and methods

Reagents and antibodies

[5,6,8,11,12,14,15-³H]PGE₂ ([³H]PGE₂; 7400 GBq/mmol) was obtained from Perkin Elmer Life Science (Boston, MA, USA). Bromosulphophthalein (BSP), bromocresol green (BCG), and PGE₂, dibutylhydroxytoluene (BHT) and d₄-PGE₂ were purchased from Sigma-Aldrich Company, Wako Pure Chemical Industries, Ltd. (Tokyo, Japan), and Cayman Chemicals & Co. (Ann Arbor, MI, USA) respectively. TGBz-T34 (TGBz) and PGB₁ (PGB₁) were provided from Ono Pharmaceutical Co., Ltd. (Osaka, Japan). Anti-human PGT rabbit polyclonal antibody was kindly obtained from Dr Michel A Fortier (Université Laval, Quebec, Canada). All other compounds and reagents were obtained from Sigma-Aldrich Company, Invitrogen, Wako Pure Chemical Industries, Ltd., or Nacalai Tesque, Inc. (Kyoto, Japan).

Cell culture

Human type 2 pulmonary epithelial A549, airway epithelial Calu-3, and mouse embryonic fibroblast 3T3-L1 cells were obtained from American Type Cell Collection (Manassas, VA, USA), and human bronchial epithelial BEAS-2B cells were purchased from DS Parma Biomedical Co., Ltd. (Osaka, Japan). All cells were grown in DMEM (Life Technology) supplemented with 10% (v/v) fetal bovine serum (Invitrogen, Life Technology), 100 units/ml penicillin, and 100 µg/ml streptomycin, at 37 °C in an atmosphere of 5% CO₂. BEAS-2B cells were incubated with different concentrations of LPS for 24 h and then mRNA expression of human PGT and IL6 was determined by quantitative real-time RT-PCR.

Enzyme immunoassay for PGE₂ determination

BEAS-2B cells were plated in 60 mm tissue culture plate, and the culture medium was replaced with medium not containing fetal bovine serum when the cells completely attached to the plate. An aliquot of extracellular medium was collected 24 h after LPS was added at the indicated concentration and diluted with enzyme immunoassay (EIA) buffer. The diluted samples were subjected to PGE₂ EIA kit (Cayman Chemicals & Co.). According to the manufacturer's protocol, PGE₂ concentration was quantified by measuring optical density at 420 nm using a micro-plate reader, ARVO MX (Perkin Elmer Life Science).

Table 1 PGT expression and uptake activity in various cell lines derived from respiratory organ. Each point represents the mean $\pm$ S.E.M. ($n=3$)

Cell lines	Origin	PGT mRNA ^a	PGE ₂ uptake ^b
3T3-L1	Mouse fibroblast	+	+
A549	Human lung carcinoma	—	—
BEAS-2B	Human bronchial epithelium	+	+
Calu-3	Human lung carcinoma	—	ND
Macrophage	Rat alveolar macrophage	—	ND

ND, not determined.

^amRNA levels of PGT (normalized to *G3PDH* expression) in 3T3-L1, A549, BEAS-2B, Calu-3, and rat alveolar macrophage were analyzed by real-time RT-PCR.

^bUptake of [³H]PGE₂ (0.5 nM) was measured for 5 min by each cells at 37 °C transport buffer (pH 7.4). The criterion is whether or not inhibited uptake of PGE₂ by PGT inhibitors.

Uptake and efflux of PGE₂ from *Xenopus laevis* oocytes

X. laevis oocytes were prepared and injected with *in vitro* copied RNA (cRNA) of PGT and then subjected to transport assays using radiolabeled PGE₂. In brief, oocytes were micro-injected with 50 nl of either deionized water or PGT cRNA solution (1 µg/µl) and placed in modified Barth's solution (MBS; 96 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.82 mM MgSO₄, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4) containing 50 µg/ml gentamicin for 3 days at 18 °C. PGT uptake was initiated by placing water- or cRNA-injected oocytes into uptake medium (ND96; 96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4) containing [³H]PGE₂ (1.0 nM) at room temperature for the indicated time. At the end of the uptake, the oocytes were washed with ice-cold MBS and intracellular accumulation of [³H]PGE₂ was measured with a liquid scintillation counter (LSC 5100, Aloka, Tokyo, Japan). For measuring efflux of PGE₂ from oocytes, water- or cRNA-injected oocytes were micro-injected with a 50 nl aliquot of concentrated [³H]PGE₂ (80 nM) solution and then the oocytes were placed with uptake medium (pH 7.4) containing unlabeled PGE₂ (10 µM) as described previously (Chan *et al.* 1998). Efflux of [³H]PGE₂ was measured for 120 min. For the inhibition study, the assay was performed in the absence (control) or the presence of inhibitor, e.g. BCG, BSP, TGBz, or PGB₁ at the indicated concentrations. The effect of these inhibitors on uptake and efflux was assessed at 60 and 90 min respectively. The kinetic analysis was conducted as described previously (Shirasaka *et al.* 2010).

RT-PCR study

Total RNA was prepared from BEAS-2B cells treated or untreated with LPS at the indicated concentration and then reverse-transcribed to cDNA. mRNA expression of PGT, IL6, and glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) was measured by quantitative RT-PCR, using gene-specific primers as follows: sense 5'-CTGTGGA-GACAATGGAATCGAG-3' and antisense 5'-CACGATCCT-GTCTTTGCTGAAG-3' for *PGT*, sense 5'-ATGTAGCCG-CCCCACACAGA-3' and antisense 5'-CATCCATCTTTT-CAGCCAT-3' for *mIL6*, and sense 5'-GGTGAAGTCG-GAGTCAACGGA-3' and antisense 5'-GAGGGATCTC-GCTCCTGGAAGA-3' for *G3PDH*. The fold change in these genes, normalized to *G3PDH* dehydrogenase (*Gapdh*) and calculated relative to the expression in wild-type mouse cells, was analyzed by 2^{-ΔΔCT} methods (Livak & Schmittgen 2001).

Figure 1

PGT protein expression in BEAS-2B cells. (A) PGT expression in BEAS-2B cells was determined by western blotting. HEK293 cells transfected empty vector and PGT cDNA were used as negative and positive control respectively. (B) Band intensity for PGT protein was subject to densitometric analysis and the normalized values with the intensity of GAPDH are shown in the bar graph. Fluorescence microscopic analysis of PGT expressed in BEAS-2B cells was performed as described in the Materials and methods section by staining cells with anti-PGT (C) or pre-absorbed anti-PGT antibody (D). The nucleus was counterstained with Hoechst 33344 (2 µg/ml). Fluorescence was observed at 63× magnification under confocal laser microscopy. Scale bar represents 20 µm.

Figure 2

Concentration-dependent effect of LPS on PGE₂ and IL6 expression in BEAS-2B cells. BEAS-2B cells were incubated in the absence or the presence of LPS (0.01, 0.03, 0.1, 0.3, and 1 µg/ml) at 37 °C. After 12 h of LPS incubation, extracellular PGE₂ levels were determined by EIA, as described in the Materials and methods section. And after 24 h of LPS incubation, mRNA levels of *IL6* were analyzed by real-time RT-PCR. The results were normalized to *G3PDH* expression. Each point represents the mean $\pm$ S.E.M. ($n=3$).

Western blot

To determine protein expression of PGT, cells were lysed in RIPA buffer (150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris at pH 8.0) by sonication. A 50 or 100 µg aliquot of the total cell lysate was separated in SDS polyacrylamide gel and then electrotransferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The blots were probed with anti-human PGT rabbit polyclonal antibody at 1:1000 dilution or with anti-G3PDH rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA) at 1:2000 dilution, followed by appropriate secondary antibodies conjugated to HRP (Invitrogen, Life Technology). Corresponding expression was detected with electrochemical luminescence assay (Wako).

Immunocytochemistry

BEAS-2B cells were plated in eight-well culture slides (BD Falcon, Franklin Lakes, NJ, USA) at a density of 1×10^5 cells/well and then fixed in 4% paraformaldehyde. Immunostaining was performed by incubating the slides with 1:20 dilution of anti-human PGT rabbit polyclonal antibody for 1 h, followed by staining with 1:400 dilution of AlexaFluor 594-conjugated anti-rabbit IgG (secondary antibody) for 1 h at room temperature. The slides were counterstained with Hoechst 33342 and then mounted with Vectashield (Vector Laboratories Burlingame, CA, USA). Antigenic peptide consisting of amino acids

622–643 of human PGT (CFISWRVKKNKEYNVQKAAGLI) was synthesized in Medical and Biological Laboratories (Nagoya, Japan). The primary antibody to PGT was incubated with the peptide at a molar ratio of 1:100 for 1 h at room temperature and then used to stain cells as a negative control. Fluorescence was examined with a confocal laser microscope LSM710 (Carl Zeiss, Hertfordshire, UK).

PGT knockdown study

BEAS-2B cells were plated in 12-well plates, and 12 h later, they were transfected with 5 nM control siRNA (Silencer Select Negative Control #1 siRNA) or siRNA mixture against *SLCO2A1* gene (Silencer Select Validated siRNAs of s13097 and s13098) with lipofectamine RNAi Max (Invitrogen) according to the manufacturer's protocol. The cells were further cultured for 48 h and then culture medium was changed to fresh medium not containing fetal bovine serum. At the same time, mRNA expression of PGT was detected according to the method described earlier. For PGE₂ assay, the medium was collected from each well (450 µl) and PGE₂ was extracted with hexane/ethyl acetate/2% formic acid solution (19:19:1, v/v/v) containing d₄-PGE₂ (10 ng/ml) as internal standard and BHT (500 ng/ml) to stabilize PGE₂ during sample preparation. The resultant organic phase was evaporated completely and reconstituted with 100 µl mobile phase 0.1% formic acid/acetonitrile (65:35, v/v) before LC/MS/MS analysis. The concentrations of PGE₂ were quantified with a LC/MS/MS system consisting a AB Sciex API 3200™ triple quadrupole mass spectrometer (AB

Figure 3

Effect of LPS on expression level of PGT and IL6 in BEAS-2B cells. BEAS-2B cells were incubated in the absence (open) or the presence (closed) of LPS (0.1 µg/ml) for various incubation periods. mRNA levels of *PGT* (A) and *IL6* (B) were analyzed by real-time RT-PCR. The results were normalized to *G3PDH* expression. * $P < 0.05$, significantly different from expression without LPS (control). Each point represents the mean $\pm$ S.E.M. ($n=3-4$).

Sciex, Foster City, CA, USA) coupled with an LC-20AD ultra-fast liquid chromatography system (Shimadzu Co., Kyoto, Japan). The mobile phase composed of the mobile phase at flow rate of 0.2 ml/min. Mercury MS (C18, 20×4.0 mm, Luna 3 μ m, Phenomenex, Torrance, CA, USA) was used as the analytical column. Injection volume was 30 μ l per sample. Samples were kept at 4 °C during the analysis. The analytes were detected using electrospray-negative ionization and monitoring the mass transitions m/z 351.1→271 for PGE₂ and m/z 355.1→275.2 for d₄-PGE₂. Analyst software version 1.4 was used for data manipulation.

Statistical analysis

Data are given as the mean of values obtained in at least three individual experiments with s.e.m. Statistical analyses were performed with the unpaired Student's *t*-test, and a probability of <0.05 ($P < 0.05$) was considered to be statistically significant.

Results

PGT expression and PGE₂ uptake activity in various cell lines derived from respiratory organ

To determine whether PGT is involved in cellular release of PGE₂ from cells, several cell lines derived from human and rodent respiratory organs were tested for *PGT* mRNA expression and PGE₂ uptake activity. As summarized in Table 1, significant mRNA expression of *PGT* was detected in 3T3-L1 and BEAS-2B cells, as these two cell lines exhibited cellular uptake of PGE₂ at a significant level; therefore, in the following experiments, release of PGE₂ was studied from BEAS-2B cells because it is derived from human bronchial epithelial cells. PGT protein expression in BEAS-2B cells was further studied by means of western blotting and immunocytochemistry. Western blotting showed that three bands between the sizes of 63 and 75 kDa, which were identical to those in HEK293 cells stably transfected with PGT cells, increased in a manner of the loaded amount of cell lysate based on densitometric analysis (Fig. 1A and B). Further fluorescence microscopic immunocytochemistry confirmed immunolocalization of PGT in the plasma membranes as well as cytoplasm in BEAS-2B cells because cells stained with pre-absorbed anti-PGT antibody with the synthesized antigenic peptide did not detect such red fluorescence (Fig. 1C and D).

Effect of LPS on extracellular PGE₂ concentration and IL6 expression in BEAS-2B cells

Effect of LPS on extracellular PGE₂ concentration and IL6 expression was examined in BEAS-2B cells (Fig. 2). When the cells were treated with LPS, extracellular concentration of PGE₂ and mRNA expression of *IL6* were elevated in a dose-dependent manner of LPS. A somewhat sigmoidal relationship was observed between extracellular concentration of PGE₂ and expression level of *IL6* mRNA. When cells were treated with 0.3 μ g/ml LPS, extracellular concentration of PGE₂ reached a plateau. Therefore,

Figure 4

Inhibitory effects of BCG, BSP, TGBz, and PGB₁ on extracellular PGE₂ and IL6 expression induced by LPS in BEAS-2B cells. BEAS-2B cells were incubated with LPS (0.1 μ g/ml) in the absence (control) or the presence of each PGT inhibitor (100 μ M BCG, 25 μ M BSP, 25 μ M TGBz, or 25 μ M PGB₁) for 24 h at 37 °C. (A) Extracellular PGE₂ levels were determined by EIA, as described in the Materials and methods section. (B) mRNA levels of *IL6* were analyzed by real-time RT-PCR. The results were normalized to *G3PDH* expression. * $P < 0.05$, significantly different from expression without inhibitors (control). Each point represents the mean $\pm$ s.e.m. ($n = 3$).

PGE₂ release was investigated in BEAS-2B cells treated with 0.1 µg/ml LPS in the following experiments. Under this condition, mRNA expression of PGT was lower in LPS-treated BEAS-2B cells than that in the untreated cells, and the expression level was unchanged in another 24 h in the treated cells (Fig. 3A). When mRNA expression of IL6 was also measured in the same manner, the expression reached the peak at 6 h in the treated cells and the level gradually decreased after 24-h exposure (Fig. 3B).

We further examined the effects of BCG, BSP, TGBz, and PGB₁, which have been characterized as inhibitors of PGT, on PGE₂ release from BEAS-2B cells for 24 h. Among them, TGBz was reported as an inhibitor with higher affinity to PGT (K_i value of 3.7 µM). As shown in Fig. 4A, BCG (100 µM) and BSP (25 µM) reduced the extracellular concentration of PGE₂ by 45.4 and 44.4% respectively while TGBz (25 µM) and PGB₁ (25 µM) increased the concentration by 71 and 19% respectively. Similar observations were made for the effect of BCG and BSP on IL6 expression in the BEAS-2B cells. *IL6* mRNA expression was attenuated in the presence of BCG and BSP by 43.9 and 94.6% respectively. By contrast, TGBz and PGB₁ stimulated IL6 expression by 218 and 342% respectively (Fig. 4B).

Effects of inhibitors on PGT-mediated PGE₂ transport

Xenopus oocytes injected with *PGT* cRNA were employed in order to study the effect of these four inhibitors on PGT-mediated PGE₂ transport across the plasma membranes. A significant increase in intracellular accumulation of PGE₂ was observed in *PGT* cRNA-injected oocytes (Fig. 5A). All the four PGT inhibitors used in this study significantly reduced PGE₂ uptake at 60 min (Fig. 5B). BCG, BSP, or PGB₁ almost abolished the uptake, while TGBz had the least effect and inhibited as much as 40% of control uptake. Inhibitory potential of these four inhibitors was also verified by their 50% inhibitory concentration (IC₅₀) to PGE₂ uptake, which of BCG, BSP, TGBz, and PGB₁ was estimated as 2.88 ± 0.38, 1.46 ± 0.20, 14.6 ± 2.6, and 0.890 ± 0.086 µM respectively (Fig. 6A, B, C and D).

In order to determine whether these inhibitors have the same potential for efflux of PGE₂, first PGT-mediated efflux of PGE₂ was studied in *Xenopus* oocytes expressing PGT. The released amount of PGE₂ from PGT-expressing oocytes was significantly greater than that from control oocytes (Fig. 7A). Furthermore, the *cis*-inhibitory effect on PGT-mediated PGE₂ efflux was examined by injecting labeled PGE₂ with or without inhibitors immediately before the assay and then PGE₂ efflux was measured in the

presence of 10 µM PGE₂ in extracellular uptake buffer. BCG (100 µM), BSP (25 µM), and PGB₁ (25 µM) reduced significantly PGT-mediated PGE₂ efflux. TGBz unlikely affected the efflux, although the tested concentration was lower than other inhibitors because of its poor solubility (Fig. 7B).

Effect of knocking down the *PGT* mRNA on release of PGE₂ from BEAS-2B cells

To determine whether PGT is involved in PGE₂ release, the *PGT* gene was knocked down in BEAS-2B cells. In cells transfected with two different siRNA mixtures, *PGT* mRNA

Figure 5

Uptake of PGE₂ was determined by *Xenopus* oocytes expressing PGT. (A) Time course of PGE₂ uptake was determined by *Xenopus* oocytes expressing PGT. Uptake of [³H]PGE₂ (1 nM) by water-injected (open) and PGT cRNA-injected (closed) oocytes was measured over 120 min at 25 °C in MBS buffer (pH 7.4). (B) Effects of BCG, BSP, TGBz, and PGB₁ on PGT-mediated uptake of PGE₂. **P* < 0.05, significantly different from uptake without inhibitors (control). Each point represents the mean ± S.E.M. (*n* = 8–10).

Figure 6

Inhibitory effects of BCG, BSP, TGBz, and PGB₁ on PGE₂ uptake by *Xenopus* oocytes expressing PGT. Uptake of [³H]PGE₂ (1 nM) by water-injected or PGT cRNA-injected oocytes was measured in the absence (control) or the

presence of BCG (A), TGBz (B), BSP (C), or PGB₁ (D) at concentration ranges of 0.1–100 μM for 60 min at 25 °C in MBS buffer (pH 7.4). Each point represents the mean ± S.E.M. (n = 8–10).

expression was reduced to $30 \pm 2\%$ of that of untransfected control cells (Fig. 8A). As arachidonic acid was added to the medium, the released amount of PGE₂ from the transfected cells for 2 h significantly decreased to 1.05 ± 0.06 ng/well from 1.55 ± 0.07 ng/well for untransfected cells.

Discussion

The intercellular concentration of PGE₂ has to be strictly regulated because PGE₂ is an inflammatory mediator. In this study, we aimed to clarify a role of PGT in PGE₂ release from cells by analyzing PGT-mediated PGE₂ efflux. Airway epithelial cells may function as both target and effector cells in response to external stimuli that cause inflammatory reactions because they can synthesize and release a variety of lipid mediators including PGE₂, 15-hydroxyeicosatetraenoic acid, PGF_{2α}, and platelet-activating factor (Churchill *et al.* 1989, Salari & Chan-Yeung 1989). Therefore, we investigated a contribution of PGT to a release of PGE₂ from human bronchial epithelial BEAS-2B cells (Table 1).

Based on the two-step model of PG signals previously reported by Nomura *et al.*, where PGE₂ is first taken up by PGT and then broken down by intracellular PGDH, PGT has been widely accepted as an influx transporter for PGE₂ such that it exerts an antiphlogistic action. By contrast, it remains unclear whether PGT contributes to the release of

PGE₂ produced by cells. In this study, we attempted to clarify a role of PGT in PGE₂ secretion under inflammatory conditions. LPS is the microbial product that activates the innate immune system (Ulevitch & Tobias 1995) and upregulates various pro-inflammatory gene expressions including COX2 (PTGS2), CXCL2, IFIT2, CCL2, CCL7, and EDN1 (Aung *et al.* 2006). We, therefore, evaluated released PGE₂ from cells treated with LPS and then observed the reduction of extracellular concentration in the presence of BCG or BSP (Fig. 4A). These two inhibitors were further demonstrated to block the efflux of PGE₂ mediated by PGT when examined in heterologously PGT-expressing *Xenopus* oocytes (Fig. 7B). On the other hand, in the presence of TGBz, which exhibited inhibitory effects on uptake but not efflux of PGE₂, the extracellular concentration of PGE₂ was slightly increased in LPS-treated BEAS-2B cells. More conclusively, a knockdown assay showed a significant contribution of PGT to PGE₂ release from BEAS-2B cells (Fig. 8). In addition, we learned that PGT protein may not be localized only at the plasma membrane but also in the cytoplasm (Fig. 1C and D). Because PGT expressed at the plasma membrane is considered to serve as an efflux transporter for PGE₂, it was hard to judge the alteration in the expression of PGT protein responsible for PGE₂ efflux in siRNA-treated cells by western blotting using their total lysates. In this study,

Figure 7

Efflux of PGE₂ by *Xenopus* oocytes expressing PGT. (A) Time course of PGE₂ efflux was determined by *Xenopus* oocytes expressing PGT. Efflux of [³H]PGE₂ (80 nM) by water-injected (open) and PGT cRNA-injected (closed) oocytes was measured over 120 min at 25 °C in MBS buffer (pH 7.4). An aliquot (50 nl) of [³H]PGE₂ was injected into oocytes and then the five oocytes were transferred to 1 ml MBS buffer. **P* < 0.05, significantly different from efflux by water oocyte. (B) Effects of BCG, BSP, TGBz, and PGB₁ on PGE₂ efflux by *Xenopus* oocytes expression PGT. Efflux of [³H]PGE₂ (80 nM) by water-injected or PGT cRNA-injected oocytes was measured for 90 min at 25 °C in MBS buffer (pH 7.4) in the absence (control) or presence of each inhibitor (100 μM BCG, 25 μM BSP, 3 μM TGBz or 25 μM PGB₁). **P* < 0.05, significantly different from efflux without inhibitors (control). Each point represents the mean ± S.E.M. (*n* = 8–10).

therefore, we simply determined mRNA expression of PGT. The difference between decreases in mRNA expression of PGT and the amount of released PGE₂ could be explained by contribution of other transporters to PGE₂ efflux. PGE₂ is a substrate of the multidrug resistance protein 4 (MRP4/ABCC4; Reid *et al.* 2003, Rius *et al.* 2005); however, Endter *et al.* reported that MRP4 mRNA was not detected in BEAS-2B cells. Therefore,

we may rule out the contribution of MRP4 on PGE₂ transport in this cell line (Endter *et al.* 2009). In addition to MRP4, other solute carriers may also be involved in the release of PGE₂ as previous studies including ours reported that at least OATP1B1 (SLCO1B1), 2B1 (SLCO2B1), 1A2 (SLCO1A2), 3A1 (SLCO3A1), and 4A1 (SLCO4A1) mediate transport of PGE₂ (Tamai *et al.* 2000, Kullak-Ublick *et al.* 2001, Adachi *et al.* 2003) and mRNA expression of OATP1A2, 1B3 (SLCO1B3), 3A1, 4A1, and 5A1 (SLCO5A1) was detected in BEAS-2B cells (Endter *et al.* 2009); however, their functional expression may be dependent on the cultural condition because of discrepancies of PGT expression between our study and their report. Hence, our siRNA study at least provides evidence of a significant contribution of PGT to PGE₂ release from BEAS-2B cells, although the contribution of other PGE₂ transporter needs to be clarified in response to LPS stimuli.

Another concern is alteration of the mechanism of PGT-mediated transport of PGE₂. According to previous reports, PGE₂ efflux was stimulated with trans-PGE₂ with a *K*_{1/2} (inhibitory constant) of 290 nM and the effect was saturated at 10 μM when PGT was expressed in *Xenopus* oocytes (Chan *et al.* 1998). According to this report, we analyzed PGE₂ secretion from *Xenopus* oocytes. In the case of PGE₂ release from the mammalian cells, it has been reported that extracellular lactate stimulates PGT-mediated PGE₂ release (Chan *et al.* 2002); however, the precise mechanism still remains to be determined. As LPS caused a significant release of PGE₂, we decided not to use PGE₂ as a trans-stimulator for PGT-mediated transport to avoid any physiological effect of PGE₂ on the EP receptor,

Figure 8

Effect of knocking down the PGT gene on release of PGE₂ from BEAS-2B cells. (A) mRNA expression of human PGT was determined by real-time RT-PCR after 48 h of siRNA transfection. (B) Extracellular PGE₂ amount was determined by LC/MS/MS, as described in the Materials and methods section. Control and siRNA-transfected BEAS-2B cells were incubated with 5 μM arachidonic acid for 2 h at 37 °C. **P* < 0.05, significantly different from control. Each column represents the mean ± S.E.M. (*n* = 5).

which may compromise the observed LPS-mediated PGE₂ release. Biological events caused by LPS may optimize or regulate endogenous trans-stimulators as PGE₂ is smoothly secreted. For a further knockdown assay, we employed LC/MS/MS to quantify PGE₂ more consistently and accurately (Fig. 8). Unfortunately, sensitivity of detection was at most 30-fold decreased; therefore, we had to add arachidonic acid to detect PGE₂ in extracellular medium at a significant level of PGE₂. In this case, the stimulation effect of LPS was not clearly observed. This could be because PG synthesis induced by arachidonic acid may be much greater than that stimulated by LPS.

LPS may alter gene expression of *PGT* gene expression in BEAS-2B cells. In this study, we observed that LPS (0.1 µg/ml) had only a marginal decrease in *PGT* mRNA expression in BEAS-2B cells (Fig. 3A). Limited information is available for the effect of LPS on *PGT* expression. One report describes that LPS did not affect rat *Pgt* expression in both mRNA and protein forms in rat cerebral endothelial cells treated with LPS for up to 24 h at the same concentration we used (Tavakoli *et al.* 2001). This implies that *PGT* expression may not be sensitive to LPS stimuli. Comparing LPS-induced reduction of *PGT* mRNA expression for 48 h with the normal condition, this could be explained as a consequence of damage by LPS-induced production of pro-inflammatory cytokines; however, this should be addressed by future studies.

LPS is a component of the Gram-negative bacterial cell membrane and has been suggested to mediate inflammation via CD14/TLR4/MD2 receptor complexes. LPS signal is transduced through MAP kinase pathways and activates various transcription factors, resulting in induction of genes encoding pro-inflammatory cytokines, e.g. IL6 and TNF-α. Independently, it is reported that BEAS-2B cells express EP₁, EP₂, and EP₄ receptors, and their exposure to PGE₂ increases *IL6* mRNA expression (Tavakoli *et al.* 2001). Interestingly, the effect of three *PGT* inhibitors (e.g. BCG, BSP, and TGBz) on *PGT*-mediated release (Fig. 7B) similarly corresponded to their effect on *IL6* mRNA expression in LPS-treated BEAS-2B cells (Fig. 4B). On the other hand, as shown in Figs 5B, 6D, and 7B, despite the inhibitory effect of PGB₁ on *PGT*-mediated PGE₂ uptake, PGB₁ elevated extracellular PGE₂ concentration and *IL6* mRNA expression in LPS-treated BEAS-2B cells. This inconsistency may be possibly explained by several mechanisms; for instance, PGB₁ may act directly on EP receptors as their agonist or antagonist, or extracellular PGB₁ may not exert an inhibition of *PGT*-mediated release. These results suggest that extracellular PGE₂, which may be adjustable by *PGT*, regulates

signal through the EP receptor in an autocrine model. Accordingly, modulation of *PGT*-mediated release may provide an antiphlogistic benefit if further study defines such a role of *PGT* under inflammatory conditions.

In conclusion, our study implies a critical role of *PGT* in regulating the extracellular concentration of PGE₂ by controlling its trans-cellular transport across the plasma membranes in cells under inflammatory conditions. Further studies should be warranted to clarify a transporter responsible for PGE₂ and its alteration in gene expression and intracellular localization under inflammatory conditions. Better understanding of a precise mechanism that determines *PGT*-mediated PGE₂ transport may provide a new rationale to efficiently control inflammatory responses induced by extracellular signaling.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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