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Prostaglandin I_2 induces apoptosis via upregulation of Fas ligand in pulmonary artery smooth muscle cells from patients with idiopathic pulmonary arterial hypertension ☆

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ABSTRACT

Background: Pulmonary vascular remodeling with idiopathic pulmonary arterial hypertension (IPAH) is associated with impaired apoptosis of pulmonary artery smooth muscle cells (PASMCs). We have reported that high-dose prostaglandin I_2 (PGI_2) therapy markedly improved hemodynamics in IPAH patients. The therapy is thought to reverse vascular remodeling, though the mechanism is unclear. The aim of this study is to assess proapoptotic effects of PGI_2 on PASMCs obtained from IPAH patients.
Methods: We investigated proapoptotic effects of PGI_2 in PAH-PASMCs by TUNEL assays, caspase-3,-7 assays and transmission electron microscopy. We examined the expression of Fas ligand (FasL), an apoptosis-inducing member of the TNF cytokine family, in PAH-PASMCs. We measured the serum FasL levels in IPAH patients treated with PGI_2.
Results: TUNEL-positive, caspase-3, 7-active cells and fragmentation of the nucleus were detected in PAH-PASMCs treated with PGI_2. The percentage of apoptotic cells induced by PGI_2 at a high concentration was higher than that induced by PGI_2 at a low concentration. PCR-array analysis revealed that PGI_2 upregulated the FasL gene in PAH-PASMCs, and we measured the FasL expression by quantitative RT-PCR and Western blotting. PGI_2 significantly increased the mRNA level of FasL by 3.98 fold and the protein level of FasL by 1.70 fold. An IP receptor antagonist inhibited the induction of apoptosis, elevation of cyclic AMP and upregulation of FasL by PGI_2. Serum FasL level had a significant positive correlation with PGI_2 dose in IPAH patients treated with PGI_2.
Conclusions: PGI_2 has proapoptotic effects on PAH-PASMCs via the IP receptor and upregulation of FasL.

1. Introduction

Idiopathic pulmonary arterial hypertension (IPAH) is a progressive disease characterized by an elevated pulmonary artery pressure (PAP) and pulmonary vascular resistance (PVR). Increased PVR is caused by pulmonary vasoconstriction and vascular remodeling by intimal and medial hypertrophy [1-3]. Pulmonary vasoconstriction and vascular remodeling are associated with a substantial number of molecules and cellular substrates, a concept referred to as the "multiple-hit theory" [4,5]. Impaired production of vasodilators such as prostaglandin I_2 (PGI_2) and NO, along with over-expression of vasoconstrictors such as endothelin-1, play an important part in the pathogenesis of IPAH [6].

Intravenous PGI_2 is the first drug to provide appreciable benefits in patients with IPAH [7]. Since PGI_2 induces relaxation of pulmonary artery smooth muscle cells (PASMCs) by stimulating the production of cyclic AMP (cAMP) [8], it was first used as a potent, short-acting vasodilator of pulmonary arteries. However, PGI_2 is expected to have a reversal remodeling effect because PGI_2 has been shown to improve hemodynamics and survival in IPAH patients without vasodilatory response to acute infusion [2]. Long-term PGI_2 therapy (mean dosage of 21 to 40 ng/kg/min) reduced mean PAP by 12% to 22% and reduced PVR by 32% to 53% compared with baseline values [9-11]. Therefore

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the appropriate dose range of PGI_2 is thought to be 25 to 40 ng/kg/min [12]. However the optimal dose of PGI_2 had been under debated and the efficacy of treatment with PGI_2 at a dose higher than 40 ng/kg/min had been unclear. We have previously reported that PGI_2 therapy at a dose higher than 40 ng/kg/min reduced mean PAP by 30% and PVR by 68% compared with baseline values in patients with IPAH [13]. We presume that high-dose PGI_2 has reversal vascular remodeling effects in addition to vasodilator effects.

Gene microarray studies have shown that lungs from patients with PAH had decreased proapoptotic/antiapoptotic gene expression ratio [14]. Bone morphogenetic proteins (BMP) -2 or -7 increased apoptosis in normal PASMCs [15]. However, apoptosis induced by BMP -2 or -7 is inhibited in PAH-PASMCs. These results indicated that PAH-PASMCs have the property of resistance to apoptosis, which causes in part pulmonary vascular remodeling.

In the present study, we tested the hypothesis that PGI_2 has proapoptotic effects on PASMCs in patients with IPAH. First we investigated the induction of apoptosis by PGI_2 in cultured PASMCs obtained from patients with IPAH. Next we measured the serum levels of Fas ligand (FasL), an apoptosis-inducing member of the TNF cytokine family, in patients with IPAH who were treated with PGI_2.

2. Material and methods

2.1. Isolation of human PASMCs

For IPAH experiments, pulmonary artery samples were obtained from 8 patients with IPAH (2 men and 6 women; mean age, 22.5 ± 11.4 years) at lung transplantation (Table 1). All patients were treated with PGI_2 before lung transplantation. The dose range of PGI_2 was 16 to 175 ng/kg/min. The range of mean PAP was 47 to 80 mmHg. For non-IPAH experiments, non-PAH-PASMCs were obtained from 3 patients (2 men and one woman; mean age, 54 ± 24.2 years). Two pulmonary artery samples were obtained from patients with lung cancer at lung lobectomy. One PASMC sample was purchased from KURABO Bio-Medical Department (Osaka, Japan). All human subject protocols were approved by the Human Ethics Committee of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, and written informed consent was obtained from all patients before the procedure. The investigation also conforms to the principles outlined in the Declaration of Helsinki. PASMCs were isolated and cultured by methods previously described [16, 17]. Peripheral pulmonary arteries smaller than 1 mm in outer diameter were disaggregated with collagenase in a water bath for 15 min at 37 °C. The adventitia and intima were removed and isolated arteries were cut into 2-mm-long sections. The cut arteries were placed in a 6-well plate with Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemental with 10% fetal bovine serum (FBS; Sigma) and 0.1 mg/mL kanamycin (Sigma). They were incubated in a humidified 5% CO_2 atmosphere at 37 °C. The culture medium was changed every 3 days. After reaching confluence, cells were subcultured with trypsin(0.05%)/ethylenediaminetetraacetic acid (EDTA). Isolated cells were identified by positive staining with antibodies against α-smooth muscle action, myosin, and smoothelin as described previously [16, 17]. Cells were used for the scheduled experiments at passage 6.

Table 1
Clinical data of patients with idiopathic pulmonary arterial hypertension used for experiments.

Patients	Age/sex	PAP (s/d/m)	PVR	CI	BNP	PGI_2 dose	Duration
1	11M	130/51/80	2629	1.9	420	26	5 yr
2	29M	68/39/47	430	1.9	450	77	6.5 yr
3	13F	111/49/67	2438	1.7	203	76	3.5 yr
4	16F	83/51/65	784	2.5	216	60	0.5 yr
5	31F	73/30/48	1199	2.1	325	175	3 yr
6	12F	99/59/72	2779	2.3	334	89	8 yr
7	43F	107/47/72	3056	2.4	622	50	4.5 yr
8	25F	90/40/58	2079	1.4	890	16	7 days

M = male; F = female;
PAP = pulmonary artery pressure (mm Hg); s/d/m = systolic/diastolic/mean; PVR = pulmonary vascular resistance (dyne·sec·cm^{-5}); CI = cardiac index (L/min/m^2); BNP = plasma concentration of brain natriuretic peptide (pg/dl); PGI_2 dose = maximum dose of PGI_2 given (ng/kg/min), and duration = period between start of PGI_2 therapy and termination of PGI_2 therapy (years or days).

2.2. Flow circuit and flow chamber

We constructed a flow circuit composed of a flow chamber, extension tubes, three-way stopcocks, a disposable syringe and a roller pump because the half-life of PGI_2 is short. The flow chamber was constructed using an I/O unit, gasket, and tissue culture dish with a diameter of 35 mm or 100 mm by methods previously described (Supplemental Figure) [18]. The extension tubes and disposable syringe filled with medium were connected to the flow chamber. The flow chamber was laid on a large dish. The circulating medium spilled over from the gap in the flow chamber. The medium that accumulated in the dish was drained by the roller pump. The flow chamber and extension tubes were maintained in an incubator at 37 °C. When PASMCs had grown until approximately 80% confluence, they were detached with trypsin (0.05%)/EDTA (0.02%). The cells were seeded in a collagen-coated dish with a diameter of 35 mm at a density of 5×10^5 cells/mL (for apoptosis assay) or in a collagen-coated dish with a diameter of 100 mm (for Western blotting and RT-PCR). After the cells had been incubated for 24 h (in DMEM supplemented with 10% FBS and 0.1 mg/mL kanamycin), two flow circuits were constructed. Culture medium (DMEM supplemented with 0.1% FBS, 25 mM HEPES and 0.1 mg/mL kanamycin) or culture medium with an IP receptor antagonist (4,5-dihydro-N-[4-[[4-(1-methyl ethoxy)phenyl]methyl] phenyl]-1H-imadazol-2-amine, CAY10441, 100 nmol/L, Cayman, Ann Arbor, MI) flowed through the main tube (6 mL/h), and PGI_2 or its diluents was infused from the side tube in each flow circuit (0.1 mL/h) under light interception. CAY10441 displays high affinity for the IP receptor. In human platelets, the receptor affinity (pK_i) is 9.3 ± 0.1 [19]. For determining dose response, two doses of PGI_2 (0.5 ng/mL and 1.0 ng/mL) were used in the study. We previously reported that high-dose PGI_2 therapy markedly improved hemodynamics in patients with IPAH [13]. The average dose of PGI_2 in the study was 107 ng/kg/min, and it was nearly 100 ng/kg/min. Dose of PGI_2 in a human of 50 kg in body weight is 5000 ng/min(calculated from the following formula: 100 ng/kg/min × 50 kg). Since normal cardiac output in adults is estimated to be 5.0 L/min, the concentration of PGI_2 in circulating blood volume is 1.0 ng/mL(calculated from the following formula: 5000 ng/min ÷ 5.0 L/min). Thus, we set a high concentration of PGI_2 in the study. The culture medium, PGI_2 and diluents were changed every 8 hours.

2.3. Evaluation of apoptosis

Transmission electron microscopy, TUNEL assay and caspase 3/7 assay were used to assess apoptosis in cultured PASMCs. Cells with definite nuclear breakage, remarkably condensed nuclear fluorescence, and significantly shrunken nuclei were defined as apoptotic cells. Transmission electron microscopy was performed with an electron microscope (H-7100: Hitachi; Tokyo, Japan). TUNEL assays were performed using an ApopTag fluorescein in situ apoptosis detection kit (Chemicon International Inc.) according to the manufacturer's instructions. Nuclear morphology was examined by labeling with prodium iodium (50 μg/mL). Caspase assay was performed using a CaspaTag in situ apoptosis detection kit (Chemicon International Inc.) according to the manufacturer's instructions. Nuclear morphology was examined by Hoechst staining. The samples were analyzed by fluorescence microscopy (Olympus IX71, Olympus Optical Co. Ltd, Tokyo, Japan). For each cover slip, 5–10 fields (with 10–30 cells in each field) were randomly selected to determine the percentage of apoptotic cells in total cells based on the morphological characteristics of apoptosis.

2.4. Apoptotic gene expression determined by RT-PCR

Apoptosis pathway-focused gene expression profiling of PAH-PASMCs treated with PGI_2 was performed with the RT^2 Profiler PCR Array System using the Human Apoptosis array (SABiosciences) according to the manufacturer's instructions. The array measures 84 key genes involved in apoptosis. Expression of mRNA was measured by RT-PCR using an ABI PRISM 7300 sequence detector system (Applied Biosystems). Quantitative RT-PCR was performed with primers for FasL (Hs.2007, SABiosciences) in combination with RT^2 SYBR® Green/ROX™ qPCR Master Mix (SABiosciences) and GAPDH (Hs99999905_m1, Applied Biosystems) in combination with TaqMan® Gene Expression Master Mix (Applied Biosystems). Expression levels were normalized against GAPDH.

2.5. Western blotting

Western blotting was performed in non-PAH-PASMCs and PAH-PASMCs (10 μg protein) as previously described [16]. Blots were incubated with rabbit polyclonal anti-FasL antibody (1:100; Ab-1, Calbiochem) and then reacted with donkey anti-rabbit secondary antibody conjugated to horseradish peroxidase (NA934, Amersham Biociences). Goat polyclonal anti-GAPDH antibody (1:200; sc-20357, Santa Cruz Biotechnology) and donkey anti-rabbit secondary antibody (AP180P, CHEMICON) were used to normalize for loading.

2.6. Measurement of cAMP

PASMCs were seeded in 6-well plates at a density of 5×10^5 cells/well. After incubation for 24 h in DMEM supplemented with 10% FBS, PGI_2 (0.1 to 10 ng/mL) was added to the culture media. After incubation for 15 minutes, cAMP was measured by a cAMP enzyme immunoassay kit (Assay Designs, Ann Arbor, MI) according to the manufacturer's instructions. The production of cAMP by PGI_2 (1.0 ng/mL) was also determined in the absence or presence of CAY10441.