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Effects of specific prostanoid EP receptor agonists on cell proliferation and intracellular Ca^{2+} concentrations in human airway smooth muscle cells

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ABSTRACT

Increased airway smooth muscle mass due to cell proliferation contributes to airway hyper-responsiveness and remodeling in patients with asthma. Prostaglandin E_2 (PGE_2) inhibits proliferation of airway smooth muscle cells, but the role of prostanoid EP receptor subtypes in mechanisms involved has not been fully elucidated yet. We investigated the effects of specific prostanoid EP receptor agonists on cell proliferation and intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in human airway smooth muscle cells. Cell numbers were assessed by mitochondria-dependent reduction of 4-[3-(4-lodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate to formazan (WST-1 assay). RT-PCR data showed that human airway smooth muscle cells express EP2, EP3, and EP4 but not EP1 receptor mRNA. PGE_2 (1 nM–1 μM) inhibited cell proliferation induced by 5% fetal bovine serum (FBS) in a concentration-dependent manner. (16S)-9-deoxy-9β-chloro-15-deoxy-16-hydroxy-17, 17-trimethylene-19, 20-didehydro PGE_2 sodium salt (ONO-AE1-259-01; EP2 receptor agonist) and 16-(3-methoxymethyl)phenyl-ω-tetranor-3,7-dithia PGE_2 (ONO-AE1-329; EP4 receptor agonist) inhibited the 5% FBS-induced cell proliferation. ONO-AE1-259-01 and ONO-AE1-329 also significantly increased the cytosolic cAMP levels. In contrast, 11,15-O-dimethyl PGE_2 (ONO-AE-248; EP3 receptor agonist) elicited an oscillatory increase in $[Ca^{2+}]_i$ but did not affect the cell growth or cAMP levels. [(17S)-2,5-ethano-6-oxo-17,20-dimethyl PGE_1] (ONO-DI-004; EP1 receptor agonist) did not affect cell growth, cAMP levels, or $[Ca^{2+}]_i$. In conclusion, PGE_2 inhibits FBS-induced cell proliferation mostly via EP2 and EP4 receptor activation and subsequent cAMP elevation. The EP3 receptor agonist causes an increase in $[Ca^{2+}]_i$ without affecting cell growth. There is no functional expression of the EP1 receptor. Research on prostanoid EP receptors may lead to novel therapeutic strategies for treatment of asthma.

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1. Introduction

The increase in airway smooth muscle mass due to enhanced proliferation and hypertrophy of airway smooth muscle cells has been implicated in the pathogenesis of airway hyper-responsiveness and remodeling in patients with asthma (Bousquet et al., 2000; Borger et al., 2006; Hirst et al., 2004; Gosens et al., 2008; Johnson et al., 2001). Thus, the pathways and mechanisms that control airway smooth muscle cell growth have been investigated intensively (Takeda et al., 2006; Tliba and Panettieri, 2009).

Prostaglandins (PGs) are mediators derived from arachidonic acid by cyclooxygenase activation (Vancheri et al., 2004; Clarke et al., 2009). PGE_2 has multiple physiological effects such as inhibiting gastric acid secretion, controlling renal blood flow, regulating female reproductive functions, and inhibiting broncho-constriction (Park and Christman, 2006; Sweatman and Collier, 1968; Vancheri et al., 2004). PGE_2 modulates various cellular functions such as contraction, cytokine production, migration, and proliferation in human airway smooth muscle cells (Ammit et al., 2000; Belvisi et al., 1998; Goncharova et al., 2003; Kume et al., 2001; Norel et al., 1999; Stewart et al., 1999). Moreover, it has been demonstrated that PGE_2 inhibits proliferation of airway smooth muscle cells (Tomlinson et al., 1995; Belvisi et al., 1998; Burgess et al., 2004; Florio et al., 1994; Johnson et al., 1995; Kassel et al., 2008; Stewart et al., 1999).

Four distinct cell-surface G-protein-coupled receptors for PGE_2 (prostanoid EP receptors), EP1, EP2, EP3, and EP4 receptors, have been identified (Breyer et al., 2001; Narumiya et al., 1999; Sugimoto and Narumiya, 2007). In general, the EP1 receptor mediates intracellular Ca^{2+} mobilization (Narumiya et al., 1999). Both EP2 and EP4 receptors are coupled to G_s, and stimulation of these receptors increases intracellular cAMP via adenylyl cyclase activation, whereas EP3 receptor inhibits adenylyl cyclase (Narumiya et al., 1999; Sugimoto and Narumiya, 2007). Nevertheless, the roles of prostanoid EP receptor subtypes in regulation of cellular functions in airway smooth muscle cells are different among

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mammalian species (Clarke et al., 2005; McGraw et al., 2006; Ndukwu et al., 1997; Norel et al., 1999; Tilley et al., 2003). Moreover, the role of prostanoid EP receptors in regulating cell proliferation of human airway smooth muscle cells is not fully elucidated yet.

This study was designed to investigate the effects of specific prostanoid-derived agonists for their respective prostanoid EP receptors (Suzawa et al., 2000) on cell proliferation and intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in human airway smooth muscle cells. We demonstrated that activation of EP2 and EP4 receptors and subsequent cAMP elevation are involved in the mechanisms underlying the inhibitory effects of PGE_2 on airway smooth muscle cell proliferation induced by fetal bovine serum (FBS). In contrast, the EP3 receptor agonist elicits intracellular Ca^{2+} mobilization but does not affect cell growth.

2. Materials and methods

2.1. Reagents

PGE_2 was obtained from Wako (Osaka, Japan). Specific prostanoid EP receptor agonists, [(17S)-2,5-ethano-6-oxo-17,20-dimethyl PGE_1], (ONO-DI-004; EP1 receptor agonist), (16S)-9-deoxy-9β-chloro-15-deoxy-16-hydroxy-17, 17-trimethylene-19, 20-didehydro PGE_2 sodium salt (ONO-AE1-259-01; EP2 receptor agonist), 11,15-O-dimethyl PGE_2 (ONO-AE-248; EP3 receptor agonist), and 16-(3-methoxymethyl)phenyl-ω-tetranor-3,7-dithia PGE_2 (ONO-AE1-329; EP4 receptor agonist), and a specific EP4 receptor antagonist 4-{4-cyano-2-[2-(4-fluoronaphthalen-1-yl) propionylamino] phenyl} butyric acid (ONO-AE3-208) were gifts from Ono Pharmaceutical Co. (Osaka, Japan) (Suzawa et al., 2000; Jones et al., 2009). A potent EP2 receptor antagonist 6-isopropoxy-9-oxoxanthene-2-carboxylic acid (AH6809) (Norel et al., 1999; Jones et al., 2009) was from Cayman (Ann Arbor, MI, USA).

2.2. Human airway smooth muscle cell culture

Primary cultures of normal human bronchial smooth muscle cells from multiple donors were obtained from Cambrex (Walkersville, MD, USA). The cells were maintained in culture medium containing 5% FBS, human recombinant epidermal growth factor (1 ng/ml), insulin (10 mg/ml), human recombinant fibroblast growth factor (2 ng/ml), gentamycin (50 mg/ml), and amphotericin B (0.05 mg/ml) (SmGM-2 BulletKit; Cambrex, Walkersville, MD, USA) in an atmosphere of 5% CO_2 and 95% air at 37 °C (Ito et al., 2008; Iwata et al., 2009; Takeda et al., 2006).

2.3. RNA isolation and RT-PCR

Total cellular RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) (Ito et al., 2010). RNA was reverse transcribed to cDNA using a Superscript III kit (Invitrogen, Carlsbad, CA, USA). PCR amplification was performed with 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min for EP2 and EP4 receptors; 35 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 1 min for EP1 receptor; or 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min for EP3 receptor and GAPDH. The sequences of the forward and reverse primers, respectively, were EP1 receptor: 5′-ACCTTCTTTGGCGGCTCT-3′ and 5′-ATGTACACCCAAGGGTCCAG-3′, EP2 receptor: 5′-TTCATCCGGCACGGGCGGACCGC-3′ and 5′-GTCAGCCTGTTTACTGGCATCTG-3′, EP3 receptor: 5′-TATGCGAGCCACATGAAGAC-3′ and 5′-TGAAGCCAGGCGAACAGCTAT-3′, EP4 receptor: 5′-CCTCCTGAGAAAGACAGTGCT-3′ and 5′-AAGACACTCTCTGAGTCCT, and GAPDH: 5′-AACGGATTTGGTCGTATTGG-3′ and 5′-TGAGTCCTTCCACGATACCA-3′. Product sizes of the EP1, EP2, EP3, EP4 receptors and GAPDH were 236 bp, 510 bp, 516 bp, 366 bp, and 498 bp, respectively.

2.4. Measurement of cell numbers

Human airway smooth muscle cells were sub-cultured at a density of 1.0×10^4 cells/cm^2 on 12-well plates (Costar3513; Corning Inc., Corning, NY, USA) in Dulbecco's modified Eagle's medium (DMEM)/F-12 culture medium (Invitrogen, Carlsbad, CA, USA) with 5% FBS in an atmosphere of 5% CO_2 and 95% air at 37 °C. Either agent or agonist was added to the cell culture medium 24 h after the cells were seeded onto 12-well plates as reported previously (Takeda et al., 2006). Cell culture medium and reagents were replaced every two days. Cell numbers were assessed by mitochondria-dependent reduction of 4-[3-(4-lodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) to formazan (WST-1 assay; Roche, Basel, Switzerland) (Takeda et al., 2006). The cells were incubated with 10% WST-1 for 2 h at 37 °C, and the amount of formazan in each well was estimated by measuring with a multi-well spectrophotometer (Wallac1420 ARVOsx; PerkinElmer, Tokyo, Japan) at a wavelength of 450 nm with a reference wavelength of 650 nm. Each experimental condition was tested in duplicate. Solvent DMSO did not affect cell growth at concentrations used (≤0.1%/vol.).

2.5. Determination of cAMP

For cAMP measurements, confluent cells that had been cultured on 6-well plates (#140675; Nunc, Roskilde, Denmark) in DMEM/F-12 medium with 5% FBS in an atmosphere of 5% CO_2 and 95% air at 37 °C were exposed to either agent for 20 min at 37 °C. The samples were treated with 0.1 M HCl for 20 min at room temperature and were homogenized. The concentrations of cAMP were estimated using a commercially available enzyme-immunoassay kit (Cayman). Each experimental condition was tested in duplicate.

2.6. Measurement of intracellular Ca^{2+} concentrations

Cells (approximately 50% confluence) grown on glass coverslips (Lab-Tek; Nunc, Rochester, NY, USA) were treated with 3 μM fura-2/AM (Dojin, Kumamoto, Japan) for 25 min at 37 °C in normal physiological solution containing (in mM): NaCl 145, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 10, and HEPES 10 (pH 7.40). After the cells were washed with normal physiological solution, the $[Ca^{2+}]_i$ was assessed by the fluorescence of fura-2 using a fluorescence microscope (Fluor20; Nikon, Tokyo, Japan) at room temperature (Ito et al., 2008; Iwata et al., 2009). Data were analyzed using a digital fluorescence imaging system (Aquacosmos; Hamamatsu Photonics, Hamamatsu, Japan). The excitation wavelengths were set at 340 and 380 nm, and the emission was collected at 510 nm by a photomultiplier. The intensity of the fura-2 fluorescence due to excitation at 340 nm (F_{340}) and 380 nm (F_{380}) was measured after subtraction of the background fluorescence, and the ratio of F_{340} to F_{380} (F_{340}/F_{380} ratio) was used as an indicator of the relative level of $[Ca^{2+}]_i$ (Ito et al., 2008; Iwata et al., 2009).

2.7. Statistical analysis

All data are expressed as means ± S.D. Analysis of variance (ANOVA) followed by the Bonferroni test for post hoc analysis was used to evaluate the statistical significance (SigmaPlot11.0, Systat Software Inc., San Jose, CA, USA). $P<0.05$ was considered statistically significant.

3. Results

3.1. Prostanoid EP receptor mRNA expression in human airway smooth muscle cells

We determined the prostanoid EP receptor mRNA expressions in human airway smooth muscle cells using RT-PCR. A549 cells, a type II alveolar epithelial cell line, were used as the control (Yano et al., 2002). Expression of EP2, EP3, and EP4 receptor mRNA was detected in human

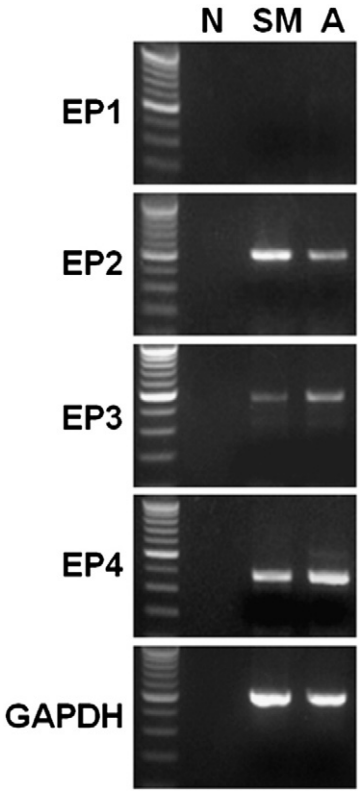

Fig. 1. Prostanoid EP receptors (EP2, EP3, and EP4 receptors) and GAPDH mRNA expression detected by RT-PCR in human airway smooth muscle cells (SM) and A549 cells (A) are shown. N indicates a negative control. EP1 receptor mRNA was not detected. The product sizes for EP1, EP2, EP3, and EP4 receptors, and GAPDH were 236 bp, 510 bp, 516 bp, 366 bp, and 498 bp, respectively.

airway smooth muscle cells and A549 cells (Fig. 1). EP1 receptor mRNA expression was not detected in either airway smooth muscle cells or A549 cells (Fig. 1). These findings are consistent with previous results in the same cell types (Clarke et al., 2005; Yano et al., 2002).

3.2. Inhibitory effect of PGE_2 on proliferation of airway smooth muscle cells

Human airway smooth muscle cells were stimulated by 5% FBS for 7 days with PGE_2 or without. Cell numbers were assessed by WST-1 assay. Absorbance of WST-1 assay, a measure of cell numbers, induced by 5% FBS for 7 days (0.900 ± 0.167) were significantly higher than those on day 1 (0.221 ± 0.050) ($P<0.001$, $n=5$). Table 1 shows the concentration-dependent effects of PGE_2 (1 nM–1 μM) on cell numbers stimulated by 5% FBS on day 7. PGE_2 (1 nM–1 μM) significantly reduced the increase in cell numbers induced by 5% FBS ($n=5$) (Table 1).

3.3. Effects of specific prostanoid EP receptor agonists on cell proliferation

The effects of specific agonists (1 nM–1 μM) for their respective prostanoid EP receptors on the 5% FBS-induced cell proliferation were examined. The cell numbers were evaluated 7 days after the cells were stimulated by 5% FBS. ONO-AE1-259-01 (EP2 receptor agonist; 100 nM and 1 μM) significantly inhibited the 5% FBS-induced cell proliferation ($n=4$) (Table 1). In addition, the augmentation of cell numbers by 5% FBS was significantly attenuated by ONO-AE1-329 (EP4 receptor agonist; 1 nM–1 μM) in a concentration-dependent manner ($n=4$) (Table 1). In contrast, ONO-DI-004 (EP1 receptor agonist) did not affect the cell proliferation stimulated by 5% FBS. The cell numbers as assessed by absorbance of WST-1 assay were not significantly different between 1 μM ONO-DI-004-treated (0.734 ± 0.106) and control (0.699 ± 0.072) groups ($n=4$). Similarly, ONO-AE-248 (EP3 receptor agonist) did not affect the 5% FBS-induced cell proliferation either. The absorbance of WST-1 assay was not significantly different between 1 μM ONO-AE-248-treated (0.720 ± 0.156) and control (0.731 ± 0.066) groups ($n=4$).

3.4. Effects of EP2 and EP4 receptor antagonists on cell proliferation

The role of EP2 and EP4 receptors in the PGE_2-induced inhibition of the cell proliferation were investigated using antagonists for EP2 or EP4 receptor. The cells were proliferated by 5% FBS for 7 days with 1 μM PGE_2 and either 1 μM AH6809 (a potent EP2 receptor antagonist) or 1 μM ONO-AE3-208 (a specific EP4 receptor antagonist). Fig. 2 shows percent inhibition of 5% FBS-induced cell growth by PGE_2 alone, PGE_2 plus either AH6809 or ONO-AE3-208. The inhibitory effects of 1 μM PGE_2 (approximately 30%) were reversed by addition of AH6809 or ONO-AE3-208 (Fig. 2). Inhibitions by PGE_2 plus AH6809 or PGE_2 plus ONO-AE3-208 were significantly less than those by PGE_2 alone ($n=4$) (Fig. 2). There is no significant difference between AH6809 plus PGE_2 and ONO-AE3-208 plus PGE_2 groups (Fig. 2).

3.5. Effects of simultaneous addition of EP2 and EP4 receptor agonists on cell proliferation

Next, the effects of simultaneous addition of 1 μM ONO-AE1-259-01 (EP2 receptor agonist) and 1 μM ONO-AE1-329 (EP4 receptor agonist) on 5% FBS-induced cell proliferation for 7 days were examined. Fig. 3 shows percent inhibition of cell growth by ONO-AE1-259-01, ONO-AE1-329, ONO-AE1-259-01 plus ONO-AE1-329, or PGE_2. The inhibitory effect of 1 μM PGE_2 on cell growth was approximately 30% (Fig. 3). The inhibitory effects of 1 μM ONO-AE1-259-01 (approximately 10%) and 1 μM ONO-AE1-329 (approximately 20%) were additive and similar to those of 1 μM PGE_2 ($n=4$) (Fig. 3). Inhibitions by ONO-AE1-259-01 plus ONO-AE1-329 or PGE_2 were significantly more than those by ONO-AE1-259-01 alone (Fig. 3). There is no significant difference between ONO-AE1-259-01 plus ONO-AE1-329 and PGE_2 groups (Fig. 3).

3.6. Effects of EP receptor agonists on intracellular cAMP levels

To confirm the involvement of cAMP in the inhibition of cell growth by prostanoid EP receptor agonists, intracellular cAMP levels

Table 1
Concentration-dependent effects of PGE_2, the specific EP2 receptor agonist ONO-AE1-259-01, and the specific EP4 receptor agonist ONO-AE1-329 on cell proliferation.

Treatment	N	Concentrations				5%FBS
		1 nM	10 nM	100 nM	1 μM	
PGE_2	5	0.815 ± 0.170[a]	0.680 ± 0.152[a]	0.621 ± 0.150[a]	0.632 ± 0.126[a]	0.900 ± 0.167
ONO-AE1-259-01	4	0.935 ± 0.103	0.890 ± 0.088	0.816 ± 0.090[a]	0.803 ± 0.121[a]	0.947 ± 0.113
ONO-AE1-329	4	0.662 ± 0.089[a]	0.614 ± 0.066[a]	0.592 ± 0.076[a]	0.638 ± 0.044[a]	0.754 ± 0.093

The cells were proliferated by 5% FBS in DMEM/F-12 medium for 7 days with either agent (1 nM–1 μM) or without. Cell numbers were assessed by absorbance of WST-1 assay. Values of absorbance of WST assay are expressed as the means ± S.D. N: the number of experiments.
[a] Significantly different from the value induced by 5% FBS ($P<0.05$, one-way repeated-measure ANOVA).

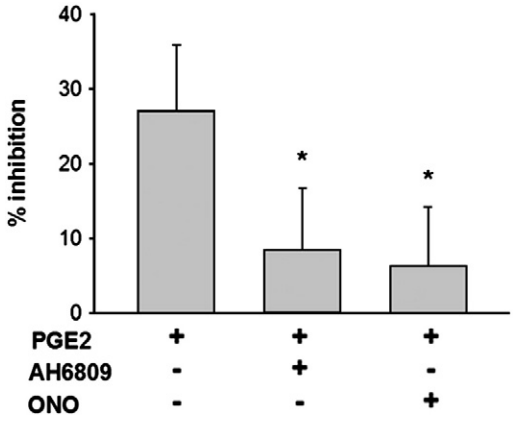

Fig. 2. Effects of a potent EP2 receptor antagonist AH6809 and the specific EP4 receptor antagonist ONO-AE3-208 on the PGE_2-induced inhibition of cell proliferation. The cells were proliferated by 5% FBS for 7 days with 1 μM PGE_2, 1 μM PGE_2 plus either 1 μM AH6809 or 1 μM ONO-AE3-208 (ONO). Bars represent the means ± S.D. of percent inhibition of cell growth assessed by WST-1 assay (n = 4). *: Significantly different from the value of induced by PGE_2 alone ($P<0.05$).

were measured. The EP2 receptor agonist ONO-AE1-259-01 (1 μM) (n = 4, $P<0.001$) and EP4 receptor agonist ONO-AE1-329 (1 μM) (n = 4, $P<0.05$) significantly increased the cAMP levels (Fig. 4). In contrast, the EP1 receptor agonist ONO-DI-004 (1 μM) or EP3 receptor agonist ONO-AE-248 (1 μM) did not affect the cAMP levels (Fig. 4).

3.7. Effects of prostanoid EP receptor agonists on intracellular Ca^{2+} concentrations

The effects of specific agonists for the respective prostanoid EP receptors on changes in $[Ca^{2+}]_i$ were investigated in fura-2-loaded human airway smooth muscle cells. A representative tracing of changes in the F_{340}/F_{380} ratio, a measure of $[Ca^{2+}]_i$, induced by the EP3 receptor agonist ONO-AE-248 is shown in Fig. 5A. Following application of ONO-AE-248 (1 μM) to the cells in normal physiological solution containing 2 mM Ca^{2+}, slow oscillatory increases in the F_{340}/F_{380} ratio were observed (Fig. 5A). In total, 71.4 ± 17.5% of the cells per field exhibited oscillatory increases in the F_{340}/F_{380} ratio greater than 0.1 by 1 μM ONO-AE-248 treatment (n = 4). In contrast, ONO-DI-004 (EP1 receptor agonist; 1 μM) did not induce oscillatory changes in the F_{340}/F_{380} ratio (Fig. 5B). Fig. 5C shows data of peak F_{340}/F_{380} ratio per field induced by specific EP receptor agonists (1 μM) (n = 4). The peak F_{340}/F_{380} ratio induced by ONO-AE-248 was significantly higher than that without inhibitors ($P<0.001$) (Fig. 5C). ONO-DI-004, ONO-AE1-259-01 (EP2 receptor agonist) or ONO-AE1-329 (EP4 agonist) did not significantly increase F_{340}/F_{380} ratio (Fig. 5C).

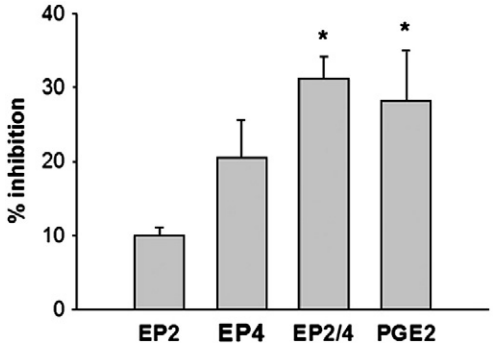

Fig. 3. Effects of simultaneous addition of EP2 receptor agonist ONO-AE1-259-01 and EP4 receptor agonist ONO-AE1-329 on cell proliferation induced by 5% FBS for 7 days. Cells were treated with 1 μM ONO-AE1-259-01 (EP2), 1 μM ONO-AE1-329 (EP4), both ONO-AE1-259-01 and ONO-AE1-329 (EP2/4) or PGE_2 (1 μM). Bars represent the means ± S.D. of percent inhibition of cell growth assessed by WST-1 assay (n = 4). *: Significantly different from the value induced by ONO-AE1-259-01 alone (EP2) ($P<0.05$).

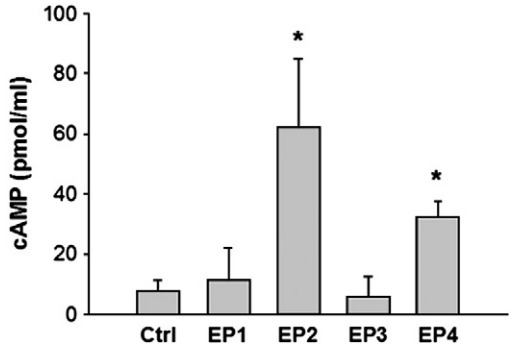

Fig. 4. Effects of EP receptor agonists, ONO-DI-004 (EP1), ONO-AE1-259-01 (EP2), ONO-AE-248 (EP3), and ONO-AE1-329 (EP4), on cAMP accumulation. Intracellular cAMP levels were assessed by an enzyme immunoassay. The concentration of either agent was 1 μM. Bars represent the means ± S.D. (n = 4). *: Significantly different ($P<0.05$) from the value induced by 5% FBS without agents (Ctrl).

3.8. EP3 receptor agonist does not increase cell proliferation

To examine whether the EP3 receptor agonist ONO-AE-248 has a mitogenic effect, the cells were treated with 1 μM ONO-AE-248 in DMEM/F-12 medium containing 0.3% FBS for 7 days. Different from 5% FBS, 0.3% FBS induced only a small increase in cell proliferation. The cell numbers as assessed by absorbance of WST-1 assay were not significantly different between ONO-AE-248-treated (0.290 ± 0.111) and untreated (0.275 ± 0.116) groups (n = 4).

4. Discussion

In the present study, a pharmacological analysis of the role of prostanoid EP receptor subtypes in regulating cell proliferation and $[Ca^{2+}]_i$ was performed in human airway smooth muscle cells. We demonstrated for the first time that the specific agonist for the EP2 or EP4 receptor inhibits cell proliferation with cAMP accumulation in human airway smooth muscle cells. PGE_2 is more potent than either an EP2 receptor agonist ONO-AE1-259-01 or EP4 receptor agonist ONO-AE1-329 alone at the same concentration in inhibiting cell growth (Fig. 3). We further found that the inhibitory effects of simultaneous addition of 1 μM ONO-AE1-259-01 and 1 μM ONO-AE1-329 on the 5% FBS-induced cell proliferation were additive and similar to those of 1 μM PGE_2 (Fig. 3). Moreover, the EP2 receptor antagonist AH6809 or the specific EP4 receptor antagonist ONO-AE3-208 reversed the inhibitory effects of PGE_2 on airway smooth muscle cell growth (Fig. 2). Therefore, activations of both EP2 and EP4 receptors are involved in the mechanisms underlying the inhibitory effects of PGE_2 on cell proliferation in human airway smooth muscle cells.

Exogenous application of PGE_2 (1 nM–1 μM) significantly inhibited the 5% FBS-induced cell proliferation in a concentration-dependent manner (Table 1). The concentrations of PGE_2 used in this study were based on previous works in airway smooth muscle cells (Belvisi, et al., 1998; Goncharova et al., 2003; Johnson et al., 1995; Kong et al., 2008). A previous study demonstrated that the PGE_2 concentrations in plasma are nano-molar levels in human normal volunteers (Ozaki et al., 1987). Importantly, local levels of PGE_2 in the lower respiratory tract are much (approximately 100 fold) higher than in the plasma (Ozaki et al., 1987), suggesting that airway smooth muscle cells may be exposed to higher concentrations of PGE_2 than the plasma level under certain conditions in vivo. It has been reported that increased cyclooxygenase-2 activities induce endogenous PGE_2 production by human airway smooth muscle cells (Belvisi et al., 1997, 1998). In our preliminary results, however, neither a nonselective cyclooxygenase inhibitor indomethacin nor a selective cyclooxygenase-2 inhibitor NS398 affected the 5% FBS-induced cell proliferation (data not shown). Thus, the effect of endogenous PGE_2 release by cyclooxygenase activation on cell growth can be ruled out in the present study.

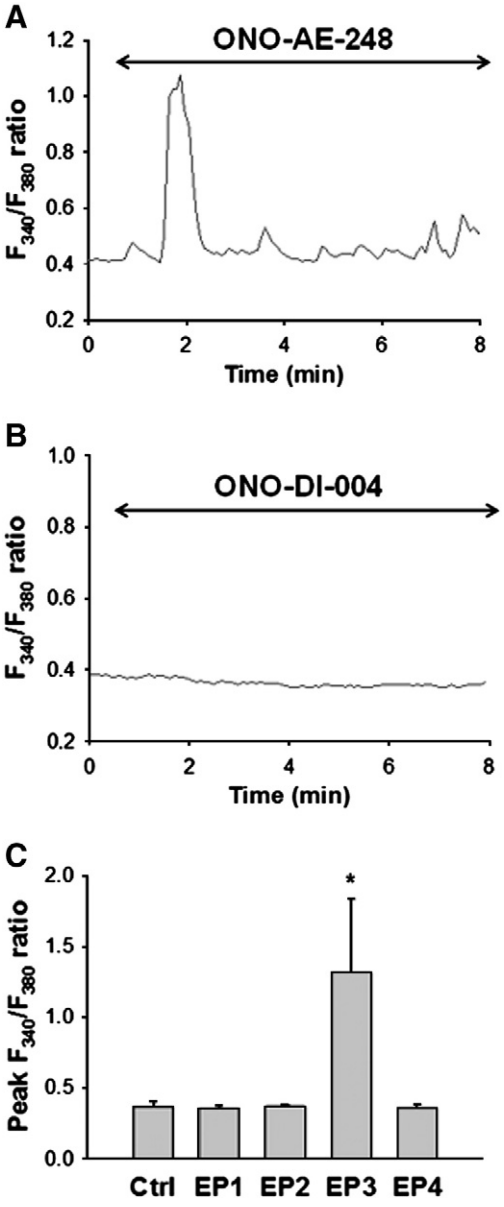

Fig. 5. Representative traces of effects of (A) EP3 receptor agonist ONO-AE-248 (1 μM) or (B) EP1 receptor agonist ONO-DI-004 (1 μM) on changes in intracellular Ca^{2+} concentrations assessed by a fura-2 fluorescence signal (F_{340}/F_{380} ratio). (C) Effects of EP receptor agonists, ONO-DI-004 (EP1), ONO-AE1-259-01 (EP2), ONO-AE-248 (EP3), and ONO-AE1-329 (EP4), on changes in F_{340}/F_{380} ratio. The means ± S.D. of peak F_{340}/F_{380} ratio induced by either agonist (1 μM) were shown (n = 4). *: Significantly different ($P<0.05$) from the control value (Ctrl) without agonist treatment.

It is established that the actions on human airway smooth muscle cells by PGE_2 occur mostly via cAMP-dependent pathways (Ammit et al., 2000; Goncharova et al., 2003; Kassel et al., 2008; Kaur et al., 2008; Panettieri et al., 1995). As shown in Fig. 1, human airway smooth muscle cells express mRNAs for EP2 and EP4 receptors, both of which are coupled to G_s (Bradbury et al., 2005; Clarke et al., 2005; Gosens et al., 2008; Narumiya et al., 1999). Indeed, the EP2 receptor agonist ONO-AE1-259-01 and EP4 receptor agonist ONO-AE1-329 significantly increased intracellular cAMP concentrations (Fig. 4). It has been demonstrated that cAMP-increasing agents such as PGE_2, β-agonist, and forskolin suppress cell proliferation in human airway smooth muscle cells (Burgess et al., 2004; Kassel et al., 2008; Stewart et al., 1999; Tomlinson et al., 1995). An increase in cAMP levels activates protein kinase A (PKA), which inhibits cell proliferation by affecting multiple pathways such as mitogen-activated protein (MAP) kinases, phosphatidyl-inositol-3-kinase (PI3K), and the JAK/STAT pathway (Gosens et al., 2008; Stork and Schmitt, 2002). In addition to PKA activation, exchange protein directly activated by cAMP (Epac), another target of cAMP, is also suggested to be involved in the inhibitory effect of cAMP on cell proliferation in airway smooth muscle cells (Gosens et al., 2008; Kassel et al., 2008). A previous report from our laboratory demonstrated that the 10% FBS-induced cell proliferation assessed by WST assay was almost completely inhibited by simvastatin via inactivating the RhoA/Rho-kinase pathway in human airway smooth muscle cells (Takeda et al., 2006). Thus, it is likely that cAMP mobilization by PGE_2 is less potent in inhibiting airway smooth muscle cell proliferation than the RhoA/Rho-kinase inactivation.

It is known that cAMP-mobilizing agents inhibit airway smooth muscle contraction. Norel et al. (1999) reported that 1 μM PGE_2 caused approximately 60% inhibition of histamine-induced contraction in human airway smooth muscle tissues. Therefore, the inhibitory effect of PGE_2 on contraction would be stronger than its anti-proliferative effect in human airway smooth muscle cells. It is suggested that EP_2 receptor is the functionally dominant EP receptor isoform but EP4 receptor is not involved in the mechanisms of relaxation by PGE_2 in human airway smooth muscle (Norel et al., 1999). We demonstrated anti-proliferative roles of both EP2 and EP4 receptors. EP4 receptor stimulation seems to be more potent in inhibiting cell proliferation than EP2 receptor stimulation (Fig. 3). These findings suggest that prostanoid EP receptors differently regulate cell growth and contraction in human airway smooth muscle cells.

EP1 receptor mRNA expression was not observed in cultured human airway smooth muscle cells (Fig. 1), consistent with previous findings (Burgess et al., 2004; Clarke et al., 2005). Indeed, the EP1 receptor agonist ONO-DI-004 had no effect on cell proliferation, cAMP levels, or $[Ca^{2+}]_i$ in human airway smooth muscle cells (Figs. 4 and 5). Both the present and previous findings demonstrate that the EP1 is not involved in the mechanisms of cell proliferation in human airway smooth muscle cells (Burgess et al., 2004). Nevertheless, possible roles of the EP1 receptor in other functions of human airway smooth muscle cannot be ruled out (Kong et al., 2008). In contrast to EP2 and EP4 receptors, it has been reported that the EP1 receptor, which is coupled to intracellular Ca^{2+} mobilization (Narumiya et al., 1999), contributes to increasing airway smooth muscle tone in mice and guinea pigs (Ito et al., 2006; Ndukwu et al., 1997; Tilley et al., 2003). In addition, EP1 receptor activation markedly reduces the broncho-dilatory function of the β_2-adrenergic receptor in mouse airway smooth muscle cells (McGraw et al., 2006). Taken together, the role of EP1 receptor in airway smooth muscle cell functions is different among mammalian species.

We confirmed that human airway smooth muscle cells also express EP3 receptor mRNA (Fig. 1), as reported previously (Burgess et al., 2004; Clarke et al., 2005; Kong et al., 2008). Furthermore, activation of the EP3 receptor by ONO-AE-248 induces oscillatory increases in $[Ca^{2+}]_i$ (Fig. 5). When 1 μM ONO-AE-248 was applied to the nominally Ca^{2+}-free solution, an increase in the F_{340}/F_{380} ratio was not observed (data not shown), indicating that Ca^{2+} influx from the extracellular side is essential for the oscillatory $[Ca^{2+}]_i$ elevation by EP3 receptor activation. Intracellular Ca^{2+} mobilization is the second messenger for contraction and cytokine production in airway smooth muscle cells (Ito et al., 2001; 2002; Iwata et al., 2009; Shiraki et al., 2009; Perez-Zoghbi et al., 2009; Sweeney et al., 2002). Moreover, it has been reported that intracellular Ca^{2+} regulates cell proliferation in airway smooth muscle cells (Perez-Zoghbi et al., 2009; Sweeney et al., 2002; Trian et al., 2007). Nevertheless, the EP3 receptor stimulation by ONO-AE-248 did not affect 5% FBS-induced cell proliferation. Furthermore, ONO-AE-248 did not increase baseline cell growth in DMEM/F-12 medium with 0.3% FBS either. Thus, it is not likely that the EP3 receptor activation affects cell proliferation in human airway smooth muscle cells. It was reported that EP3 receptor stimulation suppresses airway hyper-responsiveness by methacholine in a murine model of the ovalbumin-induced allergic asthma (Kunikata et al., 2005). In contrast, a contribution of the EP3 receptor to airway hyper-

responsiveness is also reported in mice (Tilley et al., 2003). Burgess et al. (2004) demonstrated that EP3 receptor expression as well as EP2 receptor expression in airway smooth muscle cells is increased in patients with asthma compared with control subjects. However, the role of EP3 receptor in cellular functions in human airway smooth muscle cells is still uncertain.

In summary, agonists for EP2 and EP4 receptors, which mediate intracellular cAMP accumulation, inhibited growth of human airway smooth muscle cells. In contrast, EP3 receptor activation increased $[Ca^{2+}]_i$ without affecting cell proliferation. Taken together, PGE_2 could protect against airway remodeling in patients with asthma via EP2 and EP4 receptor stimulation. Research on prostanoid EP receptors specifically EP2 and EP4 receptors may lead to novel therapeutic strategies for treatment of asthma.

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