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Prostaglandin E_2 reinforces the activation of Ras signal pathway in lung adenocarcinoma cells via EP_3

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Abstract Prostaglandin E_2 (PGE_2)-dependent effects on various cell responses are regulated by respective PGE_2 receptors (EP_1, EP_2, EP_3, EP_4) expressing in target cells. Alveolar type II cell (a main progenitor cell of lung adenocarcinoma) expressed only EP_4, while human lung adenocarcinoma cells (A549) expressed EP_3 as well as EP_4. An antagonistic effect of EP_3 against EP_4 through the modulation of cyclic AMP level is required for PGE_2-mediated activation of Ras signal pathway in A549 cells. These results suggest that the expression of EP_3 may be a critical factor for the PGE_2-mediated activation of Ras signal pathway in A549 cells. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Prostaglandin E_2; Ras; Lung adenocarcinoma; Alveolar type II cell; cAMP; EP_3

1. Introduction

Lung cancer, particularly non-small cell lung cancer (NSCLC), is one of the most common cancers and the leading cause of cancer death in Western countries [1]. NSCLC exhibiting adenocarcinoma histology is the majority of lung cancers and its *ras* genes carry mutations [2]. Furthermore, the presence of mutated *ras* genes in NSCLC is linked with a shortened patient survival [3]. These reports means that signaling pathways activated by oncogenic Ras proteins contribute to the appearance of malignant phenotypes in lung adenocarcinoma. In lung adenocarcinoma cells with Ras mutations including A549 cells, the constitutive activation of Ras signal pathway induces phospholipase A_2 and cyclooxygenase-2 (COX-2), and this induction is associated with a high generation of prostaglandin E_2 (PGE_2) in the cells [4]. This report also shows that inhibitors of PGE_2 synthesis block anchorage-independent growth of these cells. Thus, to estimate the PGE_2-dependent signals of lung adenocarcinoma cells with oncogenic Ras proteins may lead to clarification of the Ras-stimulated signaling pathways governing the appearance of malignant phenotypes in NSCLC.

There are four subtypes of PGE_2 receptors: EP_1, EP_2, EP_3 and EP_4, and different PGE_2-dependent effects are mediated by the respective receptors that are expressed in the target cells [5]. It has been well known that PGE_2 formed by the action of COX-2 participates in colon carcinogenesis [6]. A recent report has shown that PGE_2 via EP_1 mediates carcinogenic changes in the colon using knockout mice on PGE_2 receptors [7]. These PGE_2-mediated carcinogenic changes may be based on the difference of expression of EP_1 between normal and tumor cells. These reports suggest that the effects of PGE_2 on the carcinogenesis ultimately depend on the expression patterns of PGE_2 receptors in each tissue.

In this context, the present study was undertaken to estimate the difference of the expression patterns of PGE_2 receptors between A549 cells and human alveolar type II cells (a main progenitor cell of lung adenocarcinoma), and there was difference about the expression patterns. Therefore, we tried to clarify if the expressed PGE_2 receptors-regulated signals in A549 cells could contribute to stimulation of cell growth signaling in order to clarify a exact role of PGE_2 in the development of lung adenocarcinoma.

2. Materials and methods

2.1. Cell culture and treatment

A549 cells (Riken cell bank, Saitama, Japan) were routinely grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and penicillin–streptomycin. In order to arrest cell growth, 48 h starvation with low serum condition (0.02% FCS) was carried out. After the starvation, PGE_2 (Calbiochem, San Diego, CA, USA) was added to culture medium. Pertussis toxin (PTX; Calbiochem) treatment was performed for 4 h before the stimulation of PGE_2. Isobutylmethylxanthine (IBMX; Calbiochem) was added for 30 min before the stimulation. C-Br-AMP (Calbiochem) was added for 5 min before the stimulation.

2.2. RT-PCR

Messenger RNA was purified from freshly isolated human alveolar type II and cultured A549 cells as described previously [8]. Transcripts were amplified by RT-PCR using primers EP_1 (NCBI reference number 13630896): sense primer (nucleotides 284–301), antisense primer (nucleotides 1297–1317); EP_2 (NCBI 4506254): sense primer (nucleotides 168–188), antisense primer (nucleotides 760–780); EP_3 (NCBI 13638343): sense primer (nucleotides 277–297), antisense primer (nucleotide 1092–1112); EP_4 (NCBI 4506258): sense primer (nucleotides 1334–1356), antisense primer (nucleotides 1664–1687); GAPDH

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Abbreviations: cAMP, cyclic AMP; NSCLC, non-small cell lung cancer; PGE_2, prostaglandin E_2; COX-2, cyclooxygenase-2; FCS, fetal calf serum; PTX, pertussis toxin; IBMX, isobutylmethylxanthine; Erk, extracellular signal-regulated kinase; Mek-P, phosphorylated Mek; Erk-P, phosphorylated Erk; G protein, heterotrimeric guanine nucleotide-binding regulatory protein; AC, adenylate cyclase; PKA, protein kinase A

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(NCBI 7669491): sense primer (nucleotides 580–600), antisense primer (nucleotides 809–829). GAPDH was used for an internal control. After 35 cycles, PCR products were separated by electrophoresis (1.5% agarose gel) and stained with GelStar (BMA, Rockland, ME, USA). Wide-range DNA ladder (Takara, Shiga, Japan) was used as a marker for sizing the PCR products. When negative results were observed, fresh polymerase was added after 35 cycles, and the PCR was continued to 70 cycles. Additionally, in order to confirm the performance of PCR for EP_1 and EP_2, we carried out the PCR, using complementary cDNA from human breast cancer cells as a positive control (Fig. 2B).

2.3. Ras and Raf activation assay

The activation of Ras was determined according to an established method [9]. To detect the active form of Ras (Ras-GTP), we used GST-B-Raf RBD protein precoupled to glutathione–Sepharose beads. GST-B-Raf RBD protein fusion proteins were isolated as described previously [9]. Briefly, sonicated bacteria were solubilized in 1% Triton X-100 and lysates were incubated with glutathione–Sepharose overnight at 4°C. Beads were washed with PBS and stored in 50% glycerol at −20°C. Cell lysates were prepared in RIPA buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 10 mM $MgCl_2$, 25 mM NaF, 10 mM Na_3VO_4, 1 mM PMSF, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 0.1% deoxycholate). After incubation of cell lysates with the beads at 4°C for 2 h and washing three times, bound proteins eluted in sample buffer, heated for 5 min at 95°C, separated on 12.5% SDS–PAGE, transferred to nitrocellulose membrane and subjected to immunoblotting with anti-Ras antibody (Transduction Labs, Lexington, KY, USA). Raf kinase activity was determined using a single step assay for the kinase based on phosphorylation of recombinant Mek-1, detected using an activation-specific Mek antibody (New England Biolabs, Beverly, MA, USA) that recognized Mek only when specifically phosphorylated by Raf [10]. Raf was immunoprecipitated from 500 μg of lysate protein using sheep polyclonal anti-Raf antibody (UBI, Lake Placid, NY, USA). An in vitro kinase assay was performed using recombinant Mek-1 substrate (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 30°C for 30 min. The reaction mixture contained Raf-1 immunocomplex, 20 μM ATP, 500 ng of Mek-1 substrate, 20 mM NaCl, 1 mM DTT, 10 mM $MgCl_2$, 1 mM $MnCl_2$ and 20 mM Tris–HCl buffer (pH 7.4). The samples were resolved by 10% SDS–PAGE and probed with polyclonal anti-phosphorylated Mek (Mek-P; New England Biolabs), polyclonal anti-Raf (Santa Cruz Biotechnology) and monoclonal anti-Mek-1 (Transduction Labs, Lexington, KY, USA) antibodies. Detection was accomplished using ECL (Amersham, Piscataway, NJ, USA) and a cooled CCD camera-linked Cool Saver system (Atto, Tokyo, Japan). A two-dimensional densitometric evaluation of each band was performed using ATTO Image Analysis Software (ATTO). Molecular sizing was done using Rainbow molecular weight marker (Amersham). Protein concentrations were determined using DC Protein Assay (Bio-Rad, Hercules, CA, USA).

2.4. The assay of Mek and extracellular signal-regulated kinase (Erk) activations

The activation of Mek and Erk were estimated by immunoblot analysis using anti-Mek-P, phosphorylated Erk, Mek and Erk antibodies (New England Biolabs). Cell lysates were resolved in 10% SDS–PAGE, and subsequently each protein band was detected as mentioned above.

2.5. Other assay

Cell growth was determined with Quick cell proliferation assay kit using WST-1 reagent (MBL, Nagaoya, Japan). Cyclic AMP (cAMP) level was determined with an ELISA kit (Cayman Chem, Ann Arbor, MI, USA). Protein kinase A (PKA) activity was estimated with ME-SACUP Protein Kinase Assay kit (MBL, Nagoya, Japan).

2.6. Statistical analysis

Data were analyzed by one-way analysis of variance followed by Duncan's multiple-range test. A P value of 0.05 or less was considered significant.

3. Results and discussion

Initially, in order to check stimulating potential of PGE_2 on growth of A549 cells, we used a culture condition with low serum level (serum concentration, 0.02%). We selected this condition to maintain attachment of the cells for culture dishes. As shown in Fig. 1A, cell growth of A549 cells was stimulated by PGE_2 in a dose-dependent manner. In similar with cell growth, PGE_2-dependent activation of the Ras pathway in A549 cells occurred in a dose-dependent manner (Fig. 1B). These results suggest that the PGE_2-induced cell proliferation of A549 cells closely relates to the reinforcement of the activation of the Ras signal pathway. This suggestion can be supported by a previous report that the constitutive activation of Ras pathway is necessary to maintain cell proliferation in A549 cells [11]. PGE_2 receptors are specific members of a

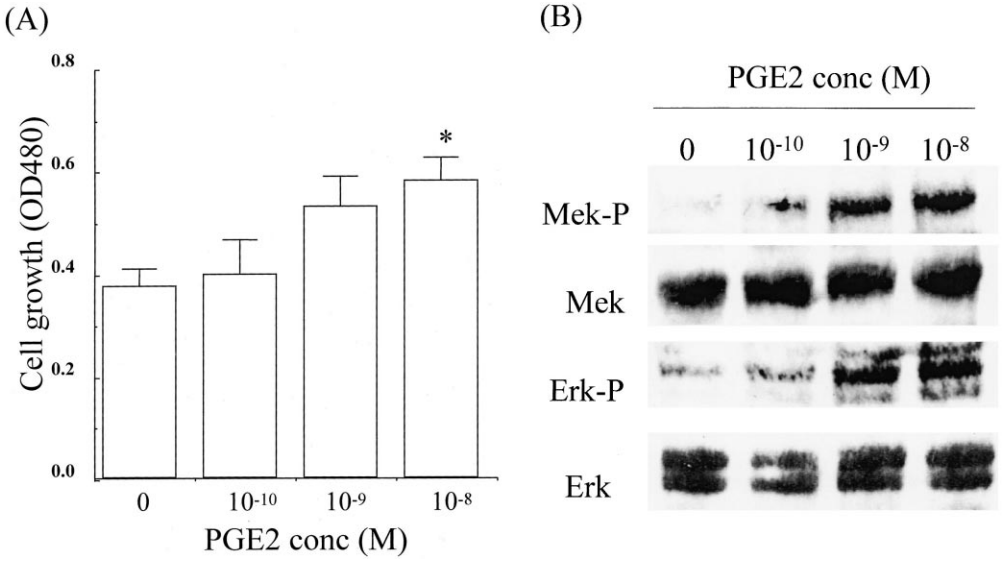

Fig. 1. Dose dependency of PGE_2 in cell growth (A) and the activation of Mek and Erk (B) in A549 cells. After 48 h starvation with low serum condition (0.02% FCS), the cells were stimulated with various concentrations of PGE_2 ($0 \sim 10^{-8}$) for 24 h and 10 min to determine cell growth and the activation of Mek and Erk, respectively. Each value indicates the mean from five samples; vertical lines indicate S.E.M. *Significantly different ($P < 0.05$) from control. The results shown are representative of three independent experiments.

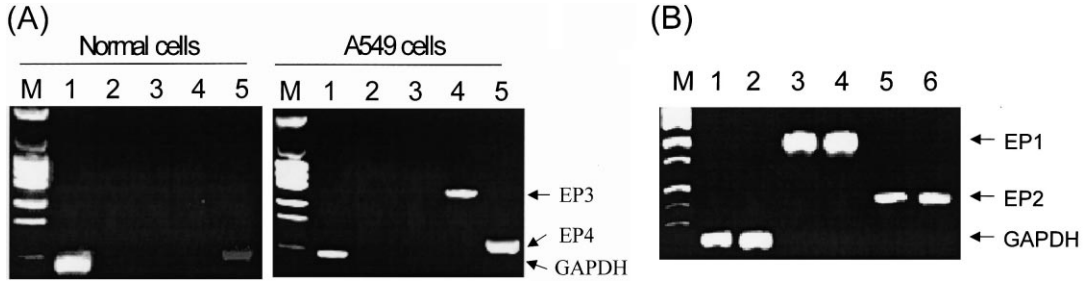

Fig. 2. RT-PCR analyses for each receptor in alveolar type II and A549 cells (A), and for EP_1 and EP_2 in human breast cancer cells (B). A: M, Size marker; 1, GAPDH; 2, EP_1; 3, EP_2; 4, EP_3; 5, EP_4. The results shown are representative of three independent experiments. B: M, Size marker; 1,2, GAPDH; 3,4, EP_1; 5,6, EP_2.

group of heterotrimeric guanine nucleotide-binding regulatory protein (G protein)-linked receptors [12], and the G protein-dependent signals govern diversity of cell responses depending on the expression patterns of the receptors in each cell [13]. Thus, it is firstly required to determine the expressed PGE_2 receptors in A549 cells in order to clarify a mechanism of PGE_2-induced activation of Ras signal pathway.

Next, by comparing the expression pattern of PGE_2 receptors in alveolar type II cells with A549 cells, we tried to estimate which receptor(s) contributed to the PGE_2-induced activation of Ras pathway in A549 cells. As shown in Fig. 2, only the expression of EP_4 was observed in alveolar type II cells, while EP_3 as well as EP_4 was expressed in A549 cells. Thus, it is possible that the expression of EP_3 in A549 cells stimulates the activation of the Ras pathway by influencing the EP_4-dependent signal pathway. In order to confirm this possibility, we investigated the effect of the expression of EP_3 on EP_4-mediated signals in A549 cells. Of the known PGE_2 receptors, EP_3 has been shown to be coupled to PTX-sensitive G protein (G_i) [12], so we used PTX as a inhibitor against EP_3 to estimate a role of EP_3 in PGE_2-stimulated activation of Erk signal pathway. As shown in Fig. 3A, TPX treatment abolished the activation of Mek caused by PGE_2 stimulation. EP_4 has been reported to be coupled to Gs protein stimulating adenylate cyclase (AC) and cAMP acts as a main signal in the EP_4-dependent signal pathway [14]. In contrast, EP_3 has been generally known to be coupled to G_i protein inhibiting AC activity [15]. At least, we detected the expression of EP3-Ia in A549 cells (data not shown), and it has been reported that the EP_3 subtype is coupled to PTX-sensitive G_i protein to inhibit cAMP level [16,17]. These reports suggest that EP_3 antagonizes the EP_4-dependent signal pathway through the regulation of cAMP level. In order to ascertain this possibility, we compared the PGE_2-induced change of cAMP level of PTX-treated cells with that of non-treated cells. The level of cAMP in the PTX-treated group showed a significant increase by PGE_2 stimulation, while the level in the non-treated group did not show any change by the stimulation (Fig. 3B). A EP_3/EP_1 agonist, sulprostone, stimulation also induced the reduction of cAMP level comparing with control (data not shown). In addition, a cAMP analogue, 8-Br-cAMP and a phosphodiesterase inhibitor, IBMX, suppressed PGE_2-dependent activation of Mek (Fig. 4). These two data further support the above possibility. Taking together, it seems that antagonistic effect of EP_3 against EP_4 leads to reinforcement of the activation of the Ras signal pathway due to the regulation of cAMP level in A549 cells stimulated with PGE_2.

Finally, we tried to determine which molecule of the Ras signal pathway was mainly regulated via the PGE_2 receptors in A549 cells after the stimulation of PGE_2. As shown in Fig. 5A,B, PTX treatment did not affect the activation of Ras, but cancelled the PGE_2-dependent activation of Raf. These results suggest that EP_3 in A549 cells reinforces the activation of the Ras signal pathway by interfering with EP_4-dependent suppression of Raf activity. Since it has been reported that Raf activity is inhibited by PKA [17], this inhibitory effect may

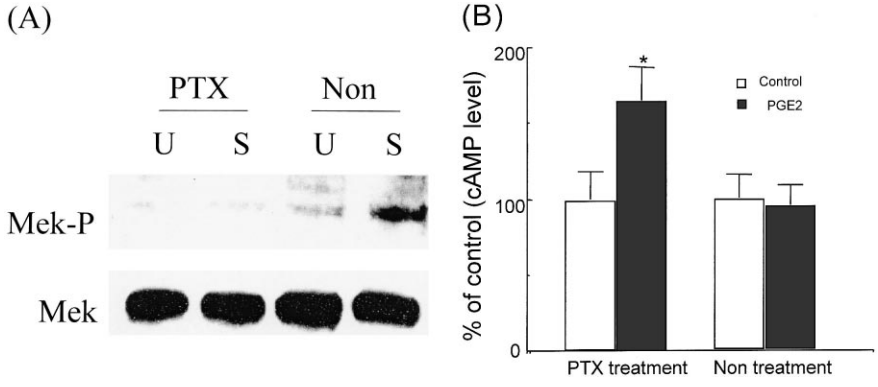

Fig. 3. The effects of PTX on the activation of Mek (A) and cAMP level (B). The cells were cultured and treated in the same condition with Fig. 1 except PTX treatment. PTX treatment (400 ng/ml) and PGE_2 stimulation (10^{-8} M) were carried out as described in Section 2. PTX, PTX-treated group; Non, non-treated group; U, unstimulated group by PGE_2; S, stimulated group by PGE_2. Each value indicates the mean from five samples; vertical lines indicate standard error. *Significantly different ($P < 0.05$) from control. The results shown are representative of three independent experiments.

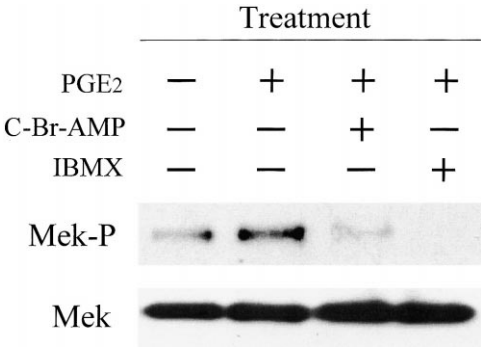

Fig. 4. The effects of c-Br-AMP and IBMX on the activation of Mek. The cells were cultured and treated in the same condition with Fig. 1 except c-Br-AMP and IBMX treatments. C-Br-AMP (100 μM), IBMX treatments (100 μM) and PGE_2 stimulation (10^{-8} M) were carried out as described in Section 2. The results shown are representative of three independent experiments.

depend on the activation of PKA. In fact, PKA activity showed a significant increase by PTX treatment (Fig. 5C).

From our present data and previous reports [5,18,19], we propose a possible mechanism on the PGE_2-dependent activation of the Ras signal pathway in A549 cells. The constitutive activation of Ras induces phospholipase A_2 and COX-2, and this induction is associated with a high generation of PGE_2 in these cells. When this produced PGE_2 stimulates EP_3, the activation of Gα-subunit of G protein coupled to EP_3 decreases the cAMP level via the inhibition of AC activity. As well, EP_3 activates the Ras signal pathway through the activation of Gβγ-subunit of G protein. In contrast, PGE_2-stimulated EP_4 induces an elevation of the cAMP level via the activation of AC activity and subsequent activation of PKA. The activated PKA causes the inactivation of the Ras signal pathway through the inhibition of Raf activity. Taking together, it seems that the expression of EP_3 in A549 cells contributes to the activation of the Ras signal pathway by suppressing the EP_4-induced increase of cAMP level via the inhibition of AC activity. Thus, the selective inhibition of the EP_3-dependent signal pathway in the lung adenocarcinoma cells is directly linked with the suppression of the Ras signal pathway. Since the blockade of the Ras signal pathway effectively suppresses the growth of tumors that constitutively activate the pathway [20,21], the idea for targeting EP_3 may lead to the development of new drugs for lung adenocarcinoma prevention and therapy.

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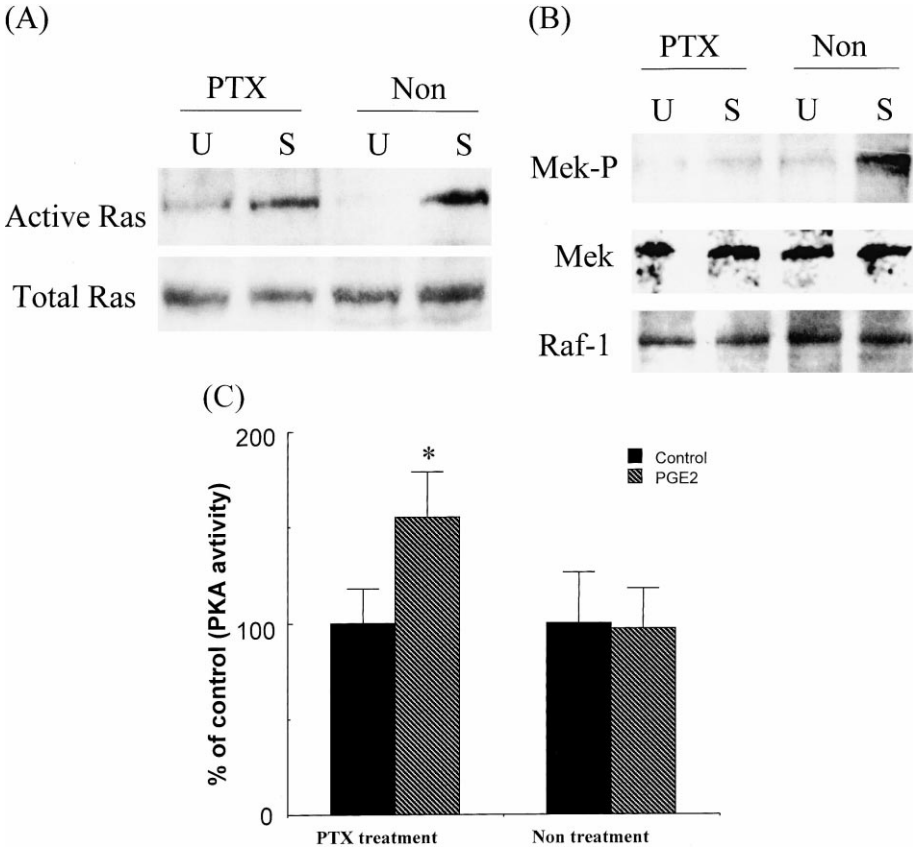

Fig. 5. The effects of PTX on the activation of Ras (A), the activities of Raf kinase (B) and PKA (C). The cells were cultured and treated in the same condition as mentioned in Fig. 3. A,B: The activation of Ras and Raf activity were determined as described in Section 2. PTX, PTX-treated group; Non, non-treated group; U, unstimulated group by PGE_2; S, stimulated group by PGE_2. C: Each value indicates the mean from five samples; vertical lines indicate standard error. *Significantly different ($P < 0.05$) from control. The results shown are representative of three independent experiments.

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