Prostaglandin E_2 upregulates β1 integrin expression via the E prostanoid 1 receptor/nuclear factor κ-light-chain-enhancer of activated B cells pathway in non-small-cell lung cancer cells

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Abstract. The prostaglandin E_2 (PGE_2) E prostanoid (EP)1 receptor shown to be associated with lung cancer cell invasion. However, the mechanism of EP1 receptor-mediated cell migration remains to be elucidated. β1 integrin is an essential regulator of the tumorigenic properties of non-small-cell lung carcinoma (NSCLC) cells. To date, little is known regarding the association between the EP1 receptor and β1 integrin expression. The present study investigated the effect of EP1 receptor activation on β1 integrin expression and cell migration in NSCLC cells. A total of 34 patients with clinical diagnosis of NSCLC and 10 patients with benign disease were recruited for the present study. The expression levels of the EP1 receptor and β1 integrin expression were studied in resected lung tissue using immunohistochemistry. A statistical analysis was performed using Stata se12.0 software. The effects of PGE_2, EP1 agonist 17-phenyl trinor-PGE_2 (17-PT-PGE_2) and the nuclear factor κ-B (NF-κB) inhibitor on β1 integrin expression were investigated on A549 cells. The expression of β1 integrin and the phosphorylation of NF-κB-p65 Ser536 was investigated by western blot analysis. Cell migration was assessed by a transwell assay. The results demonstrated that β1 integrin and EP1 receptor expression exhibited a positive correlation of evident significance in the 44 samples. The *in vitro* migration assay revealed that cell migration was increased by 30% when the cells were treated with 5 μM 17-PT-PGE_2 and that the pre-treatment of β1 integrin monoclonal antibody inhibited 17-PT-PGE_2-mediated cell migration completely. PGE_2 and 17-PT-PGE_2 treatment increased β1 integrin expression. RNA interference against the EP1 receptor blocked the PGE_2-mediated β1 integrin expression in A549 cells. Treatment with 17-PT-PGE_2 induced NF-κB activation, and the selective NF-κB inhibitor pyrrolidinedithiocarbamate inhibited 17-PT-PGE_2-mediated β1 integrin expression. In conclusion, the present study indicated that the PGE_2 EP1 receptor regulates β1 integrin expression and cell migration in NSCLC cells by activating the NF-κB signaling pathway. Targeting the PGE_2/EP1/β1 integrin signaling pathway may aid in the development of new therapeutic strategies for the prevention and treatment of this type of cancer.

Introduction

Lung cancer is one of the most common causes of cancer mortalities in men and women in the United States and worldwide (1,2). For 2013, lung cancer is expected to account for 26% of all female and 28% of all male cancer mortalities. The dismal five-year survival rate of 14-17% has shown little improvement over the past three decades (2). Although a combination of chemotherapy and radiation therapy is able to increase the survival rate, the majority of patients succumb to disease progression, which is often associated with malignant migration and metastasis (3).

Prostaglandin E_2 (PGE_2), one of most significant products of cyclooxygenase-2 (COX-2), has been suggested to be a significant cellular factor associated with cell survival and metastasis in lung cancer (4-6). PGE_2 is one of the predominant metabolic products of arachidonic acid. Previous studies revealed that PGE_2 has a significant role in a number of cancer types during tumor cell growth, migration and invasion (6-9). In non-small-cell lung carcinoma (NSCLC) cells, PGE_2 was reported to activate the extracellular-signal-regulated kinases (ERK) signaling pathway to promote cell proliferation and upregulate matrix metallaproteinase 2 expression to promote cell invasion (5,6). Targeting the cellular COX-2/PGE_2 signaling pathways provides novel therapeutic strategies for the treatment of metastasis of lung cancer (10,11).

PGE_2 has been shown to regulate tumor development and progression combining PGE_2 with E prostanoid (EP) receptors on the surface of the cell membrane and activating their

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predominant signal transduction pathways (12). Amongst these pathways, the EP1 receptor is accepted to be involved in metastasis and angiogenesis in lung cancer (13,14). However, the mechanism of the EP1 receptor-mediated cell migration remains to be elucidated in lung cancer.

Integrins are a family of transmembrane cellular receptors that mediate cell-cell and -matrix interactions. These receptors are composed of an α- and a β-subunit. Integrins of the β1 family mainly transduce signals from the extracellular matrix that modulate growth, differentiation, invasion or metastasis (15). β1 integrins have been shown to be involved in cell proliferation, adhesion and metastasis in a wide variety of human cancers, including breast, colon and ovary cancer (16-19). In NSCLC cells, an increased expression of β1 integrin was associated with cell proliferation and migration (20,21). Recently, β1 integrin was suggested as a prognostic biomarker for human lung adenocarcinoma (22).

It has been reported that fibronectin stimulates human lung carcinoma cell proliferation and diminishes apoptosis, and that this effect is mediated through integrin β1 and is associated with COX-2 expression and PGE_2 biosynthesis (23). However, the exact mechanism remains to be elucidated. In our recent studies, we found that the nuclear factor-κB (NF-κB) signaling pathway was involved in PGE_2-mediated β1 integrin upregulation in hepatocellular carcinoma cells (unpublished results from our group). The present study indicates that PGE_2 regulates β1 integrin expression and cell migration in NSCLC cells through the EP1 receptor, and that NF-κB signaling pathway may be involved in the EP1 receptor-mediated β1 integrin upregulation.

Material and methods

Reagents. The following reagents were used: The human NSCLC cell line A549 (American Type Culture Collection, Manassas, VA, USA), Dulbecco's modified Eagle medium (DMEM) and Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA), PGE_2 and 17-phenyl trinor-PGE_2 (17-PT-PGE_2; Cayman Chemical Co., Ann Arbor, MI, USA) and ammonium pyrrolidinedithiocarbamate (PDTC; Sigma-Aldrich, St. Louis, MO, USA). The protein assay kit was from Bio-Rad (Bio-Rad, Hercules, CA, USA), the electrochemiluminescence reagents were from Amersham Pharmacia Biotech (Piscataway, NJ, USA) and the 12-well transwell unit was from Costar Corning (Tewksbury, MA, USA). The following antibodies were commercially obtained: Anti-EP1 receptor rabbit polyclonal antibody (Cayman Chemical Co.), anti-β1 integrin mouse monoclonal antibody (Becton-Dickinson, Franklin Lanes, NJ, USA), anti-phosphorylated IκB mouse monoclonal antibody (#9246s) and anti-phosphorylated p65 mouse monoclonal antibody (#3036s; Cell Signaling Technology, Inc., Danvers, MA, USA), anti IκBα rabbit polyclonal antibody (#ab7217; Abcam plc, Cambridge, UK), anti-p65 rabbit polyclonal antibody (#sc-372; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti β-actin mouse monoclonal antibody (Sigma-Aldrich) and EnVisionTM single reagent (mouse, rabbit) were from DAKO (K4000, K4002, Glostrup, Denmark).

Cell lines and culture. The A549 cell line was cultured in DMEM with 10% fetal calf serum, 100 IU/ml penicillin and 100 g/ml streptomycin at 37˚C with 5% CO_2.

Patients and specimens. Primary surgical specimens were obtained from 34 patients clinically diagnosed with NSCLC, from The First Affiliated Hospital with Nanjing Medical University between January 2002 and October 2005. Out of the 34 cases, 23 were squamous cell carcinoma, 10 were adenocarcinoma and one was bronchioloalveolar carcinoma. Their ages were between 46-71 years and the average was 58 years. There were 10 benign specimens. Amongst them, four were bronchiectasis, two were inflammatory pseudotumors and four were emphysema. All specimens were collected following approval from the Human Ethics Committee (no. 2013068) of of Nanjing Medical University (Nanjing, China). Resected specimens were fixed with 10% formalin and embedded in paraffin blocks.

Immunohistochemical staining. In total, 4-μM sections of 44 tumor blocks were used for immunohistochemical analysis. The sections were deparaffinized in xylene and rehydrated in graded alcohols and water. Endogenous peroxidase activity was blocked via treatment with 3% hydrogen peroxide for 10 min. Antigen retrieval was performed by placing the slides in boiling citric acid buffer, 10 mM sodium citrate and 10 mM citric acid, for 10 min. The sections were treated with primary antibodies against β1 integrin and EP1 receptor at dilutions of 1:100 and 1:200, respectively, and incubated overnight at 4˚C. The sections were then rinsed in phosphate-buffered saline (PBS) and bound antibody was detected using the EnVision polymer technology for 60 min. Following a complete wash in PBS, the slides were developed in freshly prepared diaminobenzidine solution for 8 min and then counterstained with hematoxylin, dehydrated, air-dried and mounted with nutral balsam. PBS was used to substitute the primary antibody in the negative control. Images of the slices were captured using a Leica microscope and an image analysis system (Q550IW; Leica, Mannheim, Germany). In total, four low power views (magnification, x200) were randomly selected from each sample in a blind manner and the integrated grey level analysis was performed with Q-Win software (Leica) and presented as the mean ± standard deviation (SD).

Cell migration assays. Cell migration assays were performed in 12-well transwell units. Prior to the experiment, the lower surfaces of the membranes were coated with gelatin (1%) diluted in PBS. The A549 cells ($5x10^4$) were added to the upper chamber and 1 ml complete DMEM was added to the lower chamber of the transwell. Pharmacological agents were added at indicated times. Subsequent to incubation at 37˚C for 12 h, the cells were fixed with ethanol and then stained with 0.1% crystal violet for 30 min at room temperature. Following washing of the wells with PBS, the cells were removed from the upper surface of the membrane by wiping with a moist cotton swab. The cells which migrated to the lower surface of the membrane were solubilized with 300 μl 10% acetic acid and quantified by measuring the absorbance at 570 nm.

RNA interference. The small interfering RNAs (siRNAs) targeting the EP1 receptor (EP1R-siRNA; ID: s194727) were obtained from Ambion (Life Technologies, Grand Island, NY, USA). The sequences of the siRNAs used was ACUUCUAAGCACAACCAGAtt (sense) and

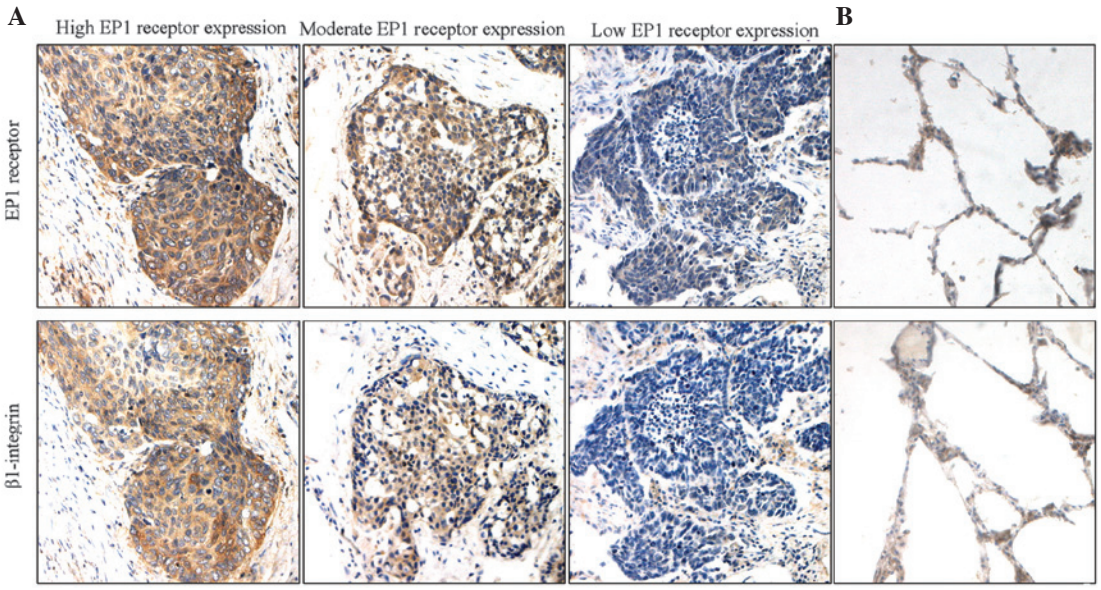

Figure 1. The expression of the EP1 receptor and β1 integrin in lung cancer tissues. (A) Immunohistochemical staining of serial sections of human NSCLC tissues reveals identical coexpression and localization of the EP1 receptor and β1 integrin. Note the cytoplasmic staining of the EP1 receptor and β1 integrin in sequential sections of the same tumor. (B) Representative immunohistochemical stains for the EP1 receptor and β1 integrin in lung tissues of benign disease. Magnification, x200. NSCLC, non-small cell lung cancer; EP1, E prostanoid 1.

5'-UCUGGUUGUGCUUAGAAGUgg-3' (antisense). The A549 cells ($4x10^4$) were plated in 12-well plates for 24 h, resulting in a 30-50% confluent cell monolayer. The cells were then transfected with the EP1R-siRNA, or a non-silencing 21-nucleotide non-coding RNA duplex as a negative control using Lipofectamine™ 2000. After 72 h, depletion of the EP1 receptor was confirmed by western blot analysis, and the cells were subsequently used for further experiments.

Western blot analysis. The cells were treated with pharmacological agents at 37˚C for various time periods, as indicated in the experiments. The cells were dissolved in lysis buffer [50 mM Tris-HCl, (pH 8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 1% nonidet™ P-40, 0.1% SDS, 100 μg/ml phenylmethanesulfonylfluoride and aprotinin] and placed on ice for 30 min. The cell lysates were sonicated on ice for at least 30 sec and then cleared by centrifugation at 120,000 x g for 30 min at 4˚C. Equal amounts of 40 μg total proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were incubated with the appropriate antibodies at 4˚C overnight with gentle agitation. The immunoreactivity was detected by electrochemiluminescence and analyzed using Image lab 4.0 analysis software (Bio-Rad).

Statistical analysis. Statistical analysis of integrated grey levels of the slices was performed using STATA se12.0 software (StataCorp, Collage Station, TX, USA). Levels of β1 integrin and EP1 receptor between NSCLC and control tissue groups were analyzed by a Shapiro-Wilk W test, to detect whether data distribution was normal and by Spearman's correlation. Other data are presented as the mean ± standard deviation. P-values were calculated using the Student's t-test for unpaired samples with MS Excel software (Microsoft Corp., Redmont, WA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Positive correlation between EP1 receptor and β1 integrin in lung cancer tissues. Immunohistochemical analysis revealed positive EP1 receptor expression in the cytoplasm in all 44 cases. In the 34 NSCLC samples tested, a strong correlation between the expression levels of β1 integrin and EP1 receptor was identified. Immunohistochemical staining of serial sections of NSCLC tissues demonstrated identical coexpression and localization of the EP1 receptor and β1 integrin. The brown staining patterns for β1 integrin and EP1 receptor expression in the cytoplasm were similar in the NSCLC tissue. The samples expressing higher levels of the EP1 receptor also exhibited greater expression of β1 integrin protein, whereas the samples with lower levels of EP1 receptor demonstrated lower or even no expression of β1 integrin (Fig. 1). Table I, shows the statistical analysis of integrated grey levels of 44 samples performed using the STATA se12.0 software (StataCorp). The β1 integrin and EP1 receptor comparisons between NSCLC and the control tissue groups were analyzed using the Shapiro-Wilk W test, and the data distribution was abnormal. Spearman's correlation assay demonstrated that the β1 integrin and EP1 receptor expression exhibited a positive correlation of evident significance in the 44 samples ($r=0.9326$ and $P<0.01$). >3/4 of the NSCLC samples exhibited significantly increased β1 integrin and EP1 receptor expression levels, compared with the benign sample groups.

EP1 receptor is involved in PGE_2-mediated cell migration and β1 integrin upregulation in lung cancer cells. The EP1 receptor has been shown to have a role in lung cancer metastasis (13). In order to assess whether β1-integrin was involved in EP1 receptor-mediated cell migration in lung cancer cells, A549 cells were pre-treated with 3 μg/ml β1 integrin monoclonal antibody (mAb) for 30 min followed by stimulation with

Table I. Correlation levels of the EP1 receptor and β1 integrin expression in human NSCLC cells and benign disease.

Patient	EP1 receptor	β1 integrin	Disease
Benign disease			
1	100.0	100.0	
2	62.7	45.7	
3	75.4	58.5	
4	111.8	126.3	
5	67.2	8.1	
6	35.9	22.5	
7	19.3	46.4	
8	35.9	25.5	
9	18.3	48.8	
10	15.9	25.7	
Non-small-cell lung cancer			
1	188.6	367.1	Adenocarcinoma
2	349.0	691.5	Adenocarcinoma
3	199.4	290.1	Squamous cell carcinoma
4	338.7	563.8	Squamous cell carcinoma
5	298.7	709.6	Squamous cell carcinoma
6	208.9	381.2	Squamous cell carcinoma
7	86.0	176.3	Squamous cell carcinoma
8	294.6	676.0	Squamous cell carcinoma
9	232.1	238.8	Adenocarcinoma
10	358.8	853.8	Squamous cell carcinoma
11	98.5	183.9	Adenocarcinoma
12	199.6	421.5	Squamous cell carcinoma
13	169.0	200.7	Squamous cell carcinoma
14	336.2	673.6	Squamous cell carcinoma
15	220.1	280.6	Squamous cell carcinoma
16	205.2	428.3	bronchioloalveolar
17	140.2	218.6	carcinoma
18	82.3	160.0	Squamous cell carcinoma
19	317.2	577.9	Adenocarcinoma
20	139.7	224.9	Squamous cell carcinoma
21	82.9	229.2	Adenocarcinoma
22	105.1	392.3	Squamous cell carcinoma
23	240.2	463.1	Squamous cell carcinoma
24	224.9	392.2	Squamous cell carcinoma
25	188.0	280.8	Adenocarcinoma
26	160.0	199.8	Adenocarcinoma
27	280.5	590.9	Adenocarcinoma
28	309.5	630.8	Squamous cell carcinoma
29	375.0	657.8	Squamous cell carcinoma
30	166.4	250.0	Squamous cell carcinoma
31	293.3	571.4	Adenocarcinoma
32	88.2	241.1	Squamous cell carcinoma
33	313.0	462.6	Squamous cell carcinoma
34	269.8	507.1	Squamous cell carcinoma

Values were normalized to the levels obtained from integrated grey levels in histochemical samples from patient 1 (100%) with benign disease. EP1, E prostanoid 1; NSCLC, non-small cell lung carcinoma.

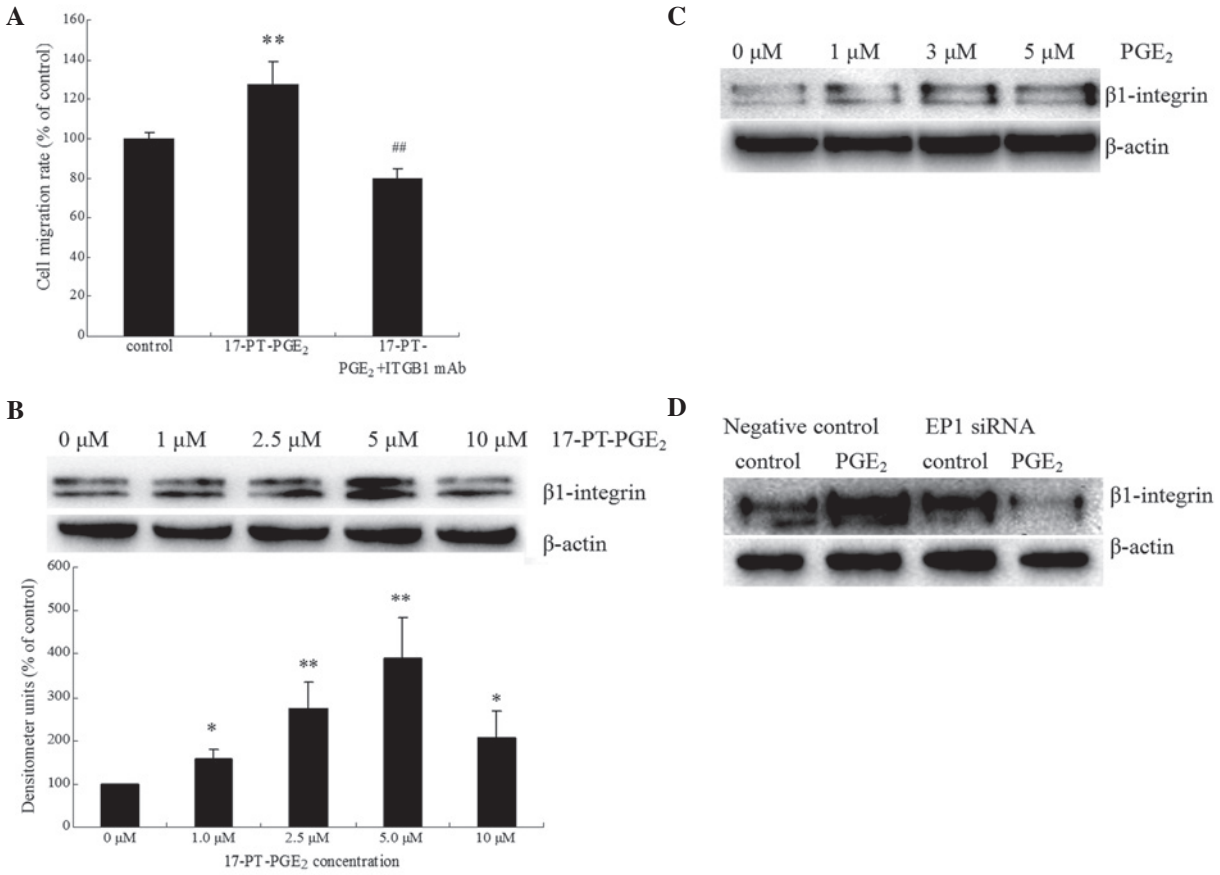

Figure 2. The EP1 receptor was involved in PGE_2-mediated cell migration and β1 integrin upregulation in A549 cells. (A) β1 integrin was involved in EP1 receptor-mediated cell migration. The results are presented as the mean ± SD from three different experiments. $^{**}P<0.01$, compared with control cells; $^{\#\#}P<0.01$, compared with cells treated with 17-PT-PGE_2. (B) Effect of PGE_2 on β1 integrin expression in A549 cells. The A549 cells were exposed to 0, 1, 3 or 5 μM PGE_2 for 24 h. Equal amounts of protein were separated by SDS-PAGE and visualized with anti-β1 integrin or β-actin antibodies. The experiments were performed three times with similar results. (C) The effect of 17-PT-PGE_2 on β1 integrin expression in A549 cells. The A549 cells were exposed to 0, 1, 2.5, 5 or 10 μM 17-PT-PGE_2 for 24 h. The relative levels of β1 integrin expression were determined using anti-β1 integrin antibody. Levels of β-actin served as a loading control and densitometric quantitation of the above blots is shown. The results are shown as the mean ± SD from three different experiments. $^{*}P<0.05$ and $^{**}P<0.01$, compared with control cells. (D) RNA interference targeting the EP1 receptor suppressed PGE_2-mediated upregulation of β1 integrin. The relative levels of β1 integrin expression were determined using anti-β1 integrin antibodies. Levels of β-actin served as a loading control. The experiments were performed three times with similar results. EP1, E prostanoid 1; PGE_2, prostaglandin E_2; 17-PT-PGE, 17-phenyl trinor prostaglandin E_2, SD, standard deviation; siRNA, small interfering RNA.

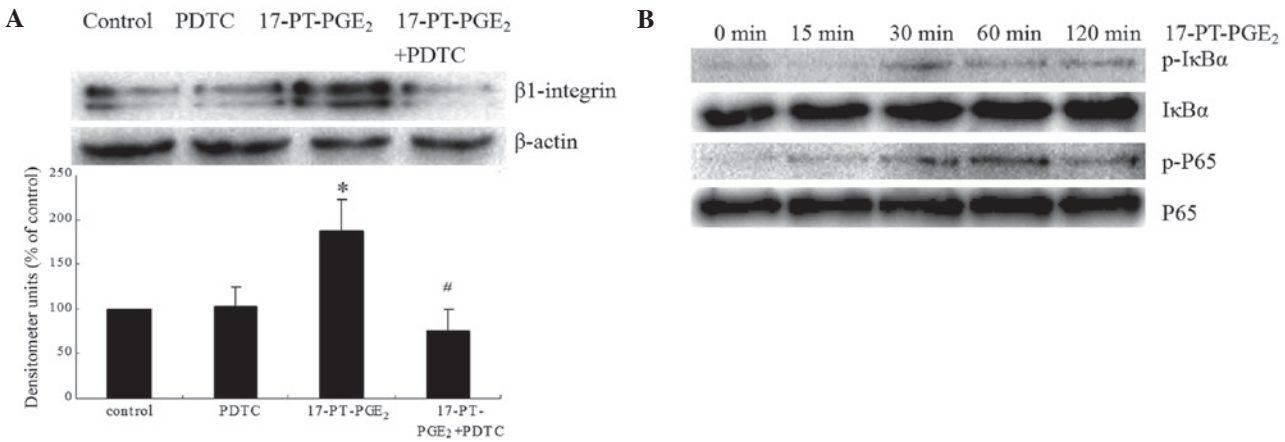

Figure 3. NF-κB was involved in EP1 receptor-mediated β1 integrin expression in A549 cells. (A) The effect of the NF-κB inhibitor on 17-PT-PGE_2-mediated β1 integrin expression in A549 cells. The A549 cells were treated with 5 μM 17-PT-PGE_2 for 24 h, with or without pre-treatment of 5 μM PDTC for 1 h. The relative levels of β1 integrin expression were determined using anti-β1 integrin antibody. β-actin was detected as a loading control and densitometric quantitation of the above blots is shown. The results are shown as the mean ± SD from three different experiments. $^{*}P<0.05$, compared with control cells; $^{\#}P<0.05$, compared with cells treated with 17-PT-PGE_2. (B) The effects of 17-PT-PGE_2 on the phosphorylation of IκBα and p65 in the A549 cells. The A549 cells were treated with 5 μM 17-PT-PGE_2 for 0, 30, 60 or 120 min. The relative levels of phosphorylation of IκBα and IκBα expression were determined using anti-phospho-IκBα and anti-IκBα antibody. The relative levels of phosphorylation of p65 and p65 expression were determined using anti-phospho-p65 antibody and anti-p65 antibody. The experiments were performed three times with similar results. NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; EP1, E prostanoid 1; 17-PT-PGE_2, 17-phenyl trinor prostaglandin E_2; PDTC, pyrrolidinedithiocarbamate; SD, standard deviation; IκBα, NF-κB inhibitor α.

17-PT-PGE_2. The *in vitro* migration activity was measured after 12 h. The cell migration was increased by 30% when the cells were treated with 5 μM 17-PT-PGE_2. The pre-treatment of β1 integrin mAb inhibited the 17-PT-PGE_2-mediated cell migration completely (Fig. 2A). Therefore, β1-integrin had a significant role in EP1 receptor-mediated cell migration in lung cancer cells.

PGE_2 increases β1 integrin expression in A549 cells. PGE_2 has previously been shown to increase β1 integrin expression in a variety of cancer types (24-26). In the present study, A549 cells were treated with 0, 1, 3 or 5 μM PGE_2 for 24 h. Fig. 2B shows that the levels of β1 integrin protein were increased in a PGE_2 concentration-dependent manner. To determine whether activation of the EP1 receptor stimulates the expression of β1 integrin, A549 cells were exposed to 0, 1, 2.5, 5 or 10 μM 17-PT-PGE_2, a selective EP1 receptor agonist. As shown in Fig. 2C, an increase in the β1 integrin protein levels was detected 24 h following 17-PT-PGE_2 treatment and the maximal response (four-fold induction) was reached at a concentration of 5 μM.

To corroborate the role of the EP1 receptor in the induction of β1 integrin expression, the A549 cells were transfected with an EP1R siRNA. As shown in Fig. 2D, depletion of the EP1 receptor did not reduce the basal levels of β1 integrin protein in A549 cells. However, the upregulation of β1 integrin expression following treatment with PGE_2 was completely blocked in the EP1R siRNA-transfected cells (Fig. 2D). The data indicate that β1-integrin was involved in the PGE_2/EP1 receptor signaling pathway in the NSCLC cells.

NF-κB signaling pathway is involved in EP1 receptor-mediated β1 integrin upregulation in lung cancer cells. Previous studies showed that a requirement for the invasion and migration of human lung cancer cells is NF-κB activation (27,28). To examine whether NF-κB activation is involved in EP1-induced β1 integrin in the NSCLC cells, an NF-κB inhibitor, PDTC, was used. Fig. 3A shows that pre-treatment with PDTC inhibited the EP1 receptor-induced β1 integrin expression in A549 cells. The phosphorylation of p65 and its upstream molecules involved in EP1 receptor activation were examined. The A549 cells were exposed to exogenous 17-PT-PGE_2 for various times. As shown in Fig. 3B, an increase in p65 phosphorylation at the Ser536 site was detected 30 min after 17-PT-PGE_2 treatment and the maximal response was reached at 60 min post-treatment with 5 μM 17-PT-PGE_2. Phosphorylation of NF-κB inhibitor α (IκB-α), an upstream molecule of p65, at the Ser32/36 site was detected 30 min after 17-PT-PGE_2 treatment, and then decreased after 60 min.

Discussion

COX-2-mediated production of PGE_2 is involved in cell growth and metastasis of a number of cancers. Previous studies indicated that COX-2 was overexpressed in numerous cancer tissues and that PGE_2 increased cancer cell growth, a process that was able to be suppressed by several COX-2 inhibitors (7,29,30). Previous studies have also demonstrated that PGE_2 induces angiogenesis (31,32) and promotes tumor cell migration and invasion (25,33). PGE_2-mediated cell growth and metastasis are coordinated by a number of proteins, including CD44 (5), epidermal growth factor receptor (EGFR) (34,35), MMP (25,36), protein kinase B (Akt) (30,37) and mitogen-activated protein kinase (6,38).

Integrins are a family of cell surface receptors for extracellular matrix proteins, including fibronectin, vitronectin, collagen and laminin (39). Integrins are heterodimeric glycoproteins, with at least 16 α- and 8 β- subunits identified thus far which are able to combine to form at least 24 different receptors, each having its own binding specificities and signaling properties (15,39). Integrins serve as adhesion receptors for extracellular matrix proteins and also transduce biochemical signals into the cell. These signaling events regulate cellular processes, including proliferation, apoptosis, migration and metastasis (40). Amongst them, β1 integrin mainly transduces signals from the extracellular matrix that modulate growth, differentiation, invasion or metastasis (15). A previous study by Zhang *et al* (41) clarified that β1 integrins are overexpressed in liver cancer and are able to mediate chemotherapy resistance. β1 integrin-mediated attachment to the extracellular matrix results in an activation of protein tyrosine kinases that protect cells from chemotherapy-induced apoptosis (42). In the context of the NSCLC cells, overexpression of β1 integrin has been reported to be associated with cell proliferation (43), migration (20,21), resistance to tyrosine kinase inhibitor gefitinib (21,44) and a poor survival rate for patients (40). Recently, β1 integrin was considered as an independent prognostic biomarker for human lung adenocarcinoma (22).

It is well understood that PGE_2 improves β1 integrin expression in a number of cancer types (24-26); A selective inhibitor of COX-2 decreased the expression of β1 integrin and suppressed cell adhesion to the extracellular matrix (45). However, the mechanism of PGE_2-mediated β1 integrin expression remains to be elucidated in different types of cancer cells.

The mechanism by which PGE_2 exerts its effects has been shown to occur by binding to four subtypes of the EP receptor. The four types of EP receptors are all expressed on the membrane surface of the NSCLC cells (14). In the present study, the malignant and the benign cases exhibited positive expression of the EP1 receptor. Of note, the immunohistochemical staining of human NSCLC tissues identified an identical coexpression and localization of the EP1 receptor and β1 integrin. Statistical analysis of integrated grey levels of 44 samples was performed using STATA se12.0 software (StataCorp). Spearman's correlation assay revealed that the EP1 receptor and β1 integrin expression exhibited a positive correlation of evident significance. The present study indicated that the EP1 receptor may be associated with β1 integrin expression in NSCLC cells.

The EP1 receptor was shown to have a significant role in the development of numerous cancer types. Previous studies by our group revealed that EP1-receptor stimulation upregulated survivin expression and focal adhesion kinase phosphorylation, and promoted cell growth and migration in hepatocellular carcinoma cells (37,46); PGE_2 stimulated fibronectin expression through the EP1 receptor/protein kinase C/c-Src pathway in primary cultured rat osteoblasts (47); PGE_2 promoted human cholangiocarcinoma cell growth and invasion through the EP1 receptor-mediated activation of the EGFR, ERK and Akt (34,38). However, thus far, little remains known about

the association between EP1 receptor activation and integrin family expression in NSCLC cells. In the present study, the effect of EP1 activation on β1 integrin expression was investigated in A549 cells. PGE_2 and the EP1 agonist 17-PT-PGE_2 increased β1 integrin expression significantly, while RNA interference targeting the EP1 receptor completely blocked PGE_2-mediated β1 integrin upregulation. These results proved that the EP1 receptor was involved in PGE_2-mediated β1 integrin expression in NSCLC cells.

Previously, the EP1 receptor has been accepted to be involved in metastasis and angiogenesis in lung cancer (13,14). However, the mechanism of EP1 receptor-mediated cell migration in NSCLC cells remains to be eluciated. The present study demonstrated that cell migration was increased following treatment with the EP1 receptor agonist, 17-PT-PGE_2. However, pre-treatment with β1 integrin monoclonal antibody inhibited 17-PT-PGE_2-mediated cell migration completely. These results indicated that β1 integrin had a significant role in EP1 receptor-mediated cell migration. The EP1 receptor may promote cell migration through increasing β1 integrin expression in NSCLC cells.

In mammals, transcription factors of the NF-κB/Rel family are able to be classified into five members: RelA (p65), c-Rel, RelB, NF-κB1 (p105/p50) and NF-κB2 (p100/p52). These are known to be significant in the inflammatory response and neoplastic development (48,49). One of the functions of Rel proteins is to bind p50 and p52, forming dimers that bind DNA and regulate transcription. The p50 (NF-κB1)/p65 (RelA) heterodimer is the most abundant form of NF-κB (50). The RelA/p65 activating signaling pathway is a critical regulator of cell growth, differentiation, apoptosis and tumorigenic transformation (48). Indeed, NF-κB is mainly activated in a number of cancer cells (50) and its activation is fundamental for the migration and invasion of lung cancer cells. The level of p65 expression was found to be increased in lung cancer tissue (28) and the NF-κB inhibitor and siRNA targeting p65 was found to downregulate E-cadherin and suppress cell migration as well as invasion in A549 cells (27).

Recently, NF-κB-p65 was found to be activated in EP1 receptor-transfected HEK293 (51) and hepatocellular carcinoma cells (unpublished results from our group). In order to identify whether NF-κB was involved in EP1 receptor-mediated β1 integrin upregulation in the NSCLC cells, the phosphorylation of p65 was detected following 17-PT-PGE_2 treatment in A549 cells. The present study revealed that the level of phospho-p65 was increased, and NF-κB was involved in the PGE_2/EP1 receptor signaling pathway in NSCLC cells. The involvement of NF-κB in EP1 receptor-mediated β1 integrin upregulation was further confirmed by using PDTC, a selective inhibitor of NF-κB, which diminished the 17-PT-PGE_2-mediated β1 integrin upregulation in A549 cells.

Overall, NF-κB is sequestered in the cytoplasm by IκB inhibitory proteins. NF-κB-activating agents are able to induce the phosphorylation of IκB proteins at Ser32 and Ser36, targeting them for rapid degradation through an ubiquitin-proteasome pathway and releasing NF-κB to enter the nucleus where it regulates gene expression (48,52). Therefore, the phosphorylation of IκBα was detected at Ser32/36 following 17-PT-PGE_2 treatment in A549 cells. The levels of phospho-IκBα were also increased following 17-PT-PGE_2 treatment for 30 min.

In conclusion, the present study demonstrated that the PGE_2 EP1 receptor upregulates β1 integrin to enhance cell migration in NSCLC cells. The NF-κB signaling pathway is involved in EP1 receptor-mediated β1 integrin expression. Thus far, little is known about the association between the EP1 receptor and β1 integrin expression. The present study provided significant novel information regarding the putative role of the EP1 receptor in β1 integrin expression in NSCLC cells and indicated that targeting the PGE_2/EP1/NF-κB/β1 integrin signaling pathway may provide novel therapeutic strategies for the prevention and treatment of this malignant disease.

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