TGF-β1 stimulates IL-8 release, COX-2 expression, and PGE₂ release in human airway smooth muscle cells

CHOONG YI FONG, LINHUA PANG, ELAINE HOLLAND, AND ALAN J. KNOX
Division of Respiratory Medicine, City Hospital, University of Nottingham,
Nottingham NG5 1PB, United Kingdom
Received 17 August 1999; accepted in final form 7 March 2000

Fong, Choong Yi, Linhua Pang, Elaine Holland, and Alan J. Knox. TGF-β1 stimulates IL-8 release, COX-2 expression, and PGE₂ release in human airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 279: L201–L207, 2000.—We have recently shown that endogenous prostanoids are critical in bradykinin-stimulated interleukin (IL)-8 release from human airway smooth muscle (ASM) cells. In this study, we tested the ability of transforming growth factor (TGF)-β1 to stimulate IL-8 release, cyclooxygenase (COX)-2 expression and PGE₂ generation in cultured human ASM cells and explored the role of COX products and COX-2 induction on IL-8 release. TGF-β1 stimulated IL-8 release, COX-2 induction, and PGE₂ generation in a concentration- and time-dependent manner. Maximal IL-8 release was achieved with 10 ng/ml of TGF-β1 after 16 h of incubation, which was inhibited by the transcription inhibitor actinomycin D and the corticosteroid dexamethasone but was not affected by the nonselective COX inhibitor indomethacin and the selective COX-2 inhibitor NS-398 despite their inhibition on TGF-β1-induced PGE₂ release. These results show for the first time that TGF-β1 stimulates IL-8 release, COX-2 expression, and PGE₂ generation in cultured human ASM cells and that PGE₂ generation is not critical for TGF-β1-induced IL-8 release. These findings suggest that TGF-β1 may play an important role in the pathophysiology of asthma.

TGF-β1 may play an important role in the pathophysiology of asthma.

Address for reprint requests and other correspondence: A. J. Knox, Division of Respiratory Medicine, City Hospital, Univ. of Nottingham, Hucknall Rd., Nottingham NG5 1PB, UK (E-mail: alan.knox@nottingham.ac.uk).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
MATERIALS AND METHODS

Cell culture. Primary cultures of human ASM cells were prepared from explants of ASM according to methods previously reported by our group (22, 23). Human tracheae were obtained from two postmortem individuals within 12 h of death. The two patients were both male; one was an ex-smoker who died of a ruptured arterial aneurysm and hematoperitonium at the age of 70, and the other was a nonsmoker who died of myeloma at the age of 82. There was no evidence of airway disease as determined by history and macroscopic examination of the trachea and lungs. Cells at passages 3 and 4 from the two donors were used for all experiments. Pang and Knox (22) have previously shown that cells grown in this manner depict the immunohistochemical and light-microscopic characteristics of typical ASM cells.

Experiment protocol. The cells were plated at a density of 2 × 10⁴ cells/well in 24-well culture plates and cultured to confluence in 10% FCS (Seralab, Crownly Down, UK)-DMEM (Sigma, Poole, UK) in humidified 5% CO₂-95% air at 37°C and growth arrested in FCS-free DMEM for 24 h before the experiments. Immediately before each experiment, fresh FCS-free DMEM containing TGF-β1 (10 ng/ml; Sigma) was added. In the time-course experiments, the cells were incubated with TGF-β1 (10 ng/ml) for 30 min to 24 h. In the concentration-response experiments, the cells were incubated for 16 h with 0.01–100 ng/ml of TGF-β1. The culture media were harvested at the indicated times and stored at −20°C before analysis with ELISA for IL-8 and with RIA for PGE₂ as a content of a representative of prostanoic generation. Protein extraction was performed before Western blot analysis to measure COX-1 and COX-2 expression. Protein was extracted by incubating the cells for 5 min with 50 μl/well of protein extraction buffer (0.9% NaCl, 20 mM Tris-HCl, pH 7.6, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 0.01% leupeptin; all from Sigma) and shaking gently. The protein extraction buffer was then harvested and stored at −20°C for subsequent Western blot analysis. To test the mechanisms involved in the effect of TGF-β1, the nonselective COX inhibitor indomethacin (Indo), the gene transcription inhibitor actinomycin D (Act D), the anti-inflammatory steroid dexamethasone (Dex; all from Sigma), the selective COX-2 inhibitor NS-398 (1-[2-cyclohexyloxy]-4-nitrophe-noxyphenyl)-methanesulfonamide; Cayman Chemical, Ann Arbor, MI), or vehicle control (DMSO) was added at various concentrations 1 h before the addition of TGF-β1.

IL-8 assay. The concentration of IL-8 in the culture medium was determined by ELISA (CLB, Amsterdam, The Netherlands) according to the manufacturer’s instructions. Briefly, ELISA plates were coated overnight at room temperature with 200 μl of anti-human IL-8 coating antibody that had been diluted in 0.1 M carbonate-bicarbonate buffer (pH 9.6). The plates were then washed five times with PBS (pH 7.2–7.4) containing 0.005% Tween 20 and blocked for 1 h at room temperature with 200 μl of blocking buffer. The plates were washed again, and 100 μl of samples containing standard amounts of recombinant human IL-8 as well as study samples obtained from harvested culture medium (diluted 1:5 with dilution buffer) were added in duplicate to individual wells and incubated at room temperature for 1 h. After five washes, 100 μl of biotinylated IL-8 antibody diluted in dilution buffer were added for 1 h. After another five washes, 100 μl of streptavidin-horseradish peroxidase (HRP) conjugate that had been diluted 1:10,000 in dilution buffer were added for 30 min. After a final wash, 100 μl of the substrate buffer containing the HRP substrate tetramethylbenzidine dihydrochloride and hydrogen peroxide in 0.05 M phosphate-citrate buffer (pH 5.0) were added for 30 min in the dark, and color developed in proportion to the amount of IL-8 present. The reaction was stopped by adding 100 μl of stop solution (1.8 M sulfuric acid). The degree of color that had been generated was determined by measuring the optical density at 450 nm in a Dynatech MR5000 microplate reader (Billinghurst, UK). The standard curve was linearized and subjected to regression analysis. The IL-8 concentration of unknown samples was extracted using the standard curve. The results are expressed as picograms per milliliter. The sensitivity of the ELISA kit in our study was at least 5 pg/ml, which was consistent with the manufacturer’s specifications. According to the kit insert, the anti-IL-8 antibody does not cross-react with IL-1 through IL-7, IL-9 through IL-11, tumor necrosis factor, interferon-γ, granulocyte-macrophage colony-stimulating factor, and RANTES. All the reagents used were supplied by the ELISA kit manufacturer with the exception of the HRP substrate tetramethylbenzidine dihydrochloride, which was obtained from Sigma.

Western blot analysis. COX-1 and COX-2 expression were assessed by Western blotting. The protein concentration of cell extracts was determined with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hemel Hempstead, UK). Sufficient aliquots of sample (30 μg protein/track) were mixed 1:1 with sample buffer [20 mM Tris-HCl, pH 6.8, 20% glycerol, 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, and 0.025% bromphenol blue; all from Sigma] and boiled for 5 min before electrophoresis. Electrophoresis was performed on these samples on a 20 × 20-cm 7.5% SDS-polyacrylamide gel (45 mA, 5 h). The separated proteins were then electroblotted (150 V, 3 h) to pure nitrocellulose membrane (Gelman Sciences, Northampton, UK). The blot was blocked for 2 h at 4°C in blocking reagent [8% fat-free dried milk powder in PBS, pH 7.4, with 0.3% Tween 20 (PBS-T)], incubated with primary monoclonal anti-human COX-2 antibody (1:2,000 in blocking reagent; Cayman Chemical) for 2 h at room temperature before being washed with PBS-T and incubated with rabbit anti-mouse IgG coupled with HRP (1:2,000 in blocking reagent; Sigma) for 1 h at room temperature. Semiquantitative staining was achieved by using enhanced chemiluminescence detection. This detection was performed by washing the blot with PBS-T, incubating it with the SuperSignal CL-HRP substrate system (Pierce, Rockford, IL) for 1 min, and finally exposing it to Hyperfilm ECL (Amersham Life Science, Little Chalfont, UK). The positions and molecular masses of COX-2 and COX-1 were validated by reference to rainbow-colored molecular mass markers (Amersham Life Science). Reprobing of COX-1 was carried out by incubating the membrane in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl) at 50°C for 30 min with occasional agitation, washing the membrane in a large volume of PBS-T, blocking the membrane for 2 h in blocking reagent, and then following the steps described above to detect COX-1 with monoclonal anti-ovine COX-1 antibody (with cross-reactivity to human COX-1, 1:2,000 in blocking reagent; Cayman Chemical).

PGE₂ assay. Assays were performed in duplicate and are expressed as picograms per milliliter. Doubling dilutions of the PGE₂ standard (Sigma) over the concentration range 1,000–7.5 pg/100 ml were made up in assay buffer (1 g of gelatin, 6.1 g of NaCl, 10.8 g of K₂HPO₄, 1.7 g of KH₂PO₄, and 0.7 g of Na₃cit) in 500 ml of distilled H₂O and used to construct the standard curve. Prepared standards and samples were then incubated overnight with [H]PGE₂ (Amer- sham) and rabbit PGE₂ antiserum (1:8 with assay buffer; Sigma) in assay buffer at 4°C. The bound labeled antibody
complexes were then separated with the use of dextran-coated charcoal. Samples and standard were then centrifuged for 15 min at 3,500 rpm (4°C) to precipitate the unbound labeled PGE$_2$. The supernatants were then transferred into scintillation vials, and 10 ml of scintillation emulsifier cocktail (Packard, Pangbourne, UK) were added to each vial. The samples were then counted with a $\beta$-counter (5 min/vial). The anti-PGE$_2$ antiserum (Sigma) in our hands had negligible cross-reactivity (22).

**Cell viability.** The toxicity of all chemicals used in this study [e.g., TGF-$\beta_1$, COX inhibitors, Act D, Dex, and drug vehicles DMSO and ethanol (final concentration 1% vol/vol; Sigma)] to human ASM cells was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. After a 24-h incubation with the chemicals, 20 ml of 5 mg/ml of MTT were added to the culture medium in 96-well plates and incubated for 1 h at 37°C. After the medium was removed, 200 µl of DMSO were added to solubilize the blue-colored tetrazolium, the plates were shaken for 5 min, and the optical density values at 550 nm were read in a microplate reader. Viability was set as 100% in control cells.

**Statistical analysis.** Data are expressed as means ± SE from $n$ determinations. The statistical analysis was performed with software from SPSS (34). A one-way ANOVA and/or an unpaired, two-tailed Student’s $t$-test was used to determine the significant differences between the means. The results were adjusted for multiple testing with Bonferroni’s correction. $P$ values < 0.05 were accepted as significant.

**RESULTS**

**Effect of TGF-$\beta_1$ on IL-8 release.** Time-course and concentration-response experiments were conducted to investigate the effect of TGF-$\beta_1$ on IL-8 release. In the time-course experiments (Fig. 1A), there was a slight increase in IL-8 release from control cells over the 24-h incubation (from 5.2 ± 2.1 pg/ml at 30 min to 9.9 ± 0.6 pg/ml at 24 h). In cells stimulated with TGF-$\beta_1$, there was a time-dependent increase in IL-8 release. A significant difference in IL-8 release after stimulation with TGF-$\beta_1$ was observed after 8 ($P < 0.01$), 16 ($P < 0.001$), and 24 ($P < 0.01$) h. Because the highest IL-8 release was obtained at 16 h (133.7 ± 16.9 pg/ml), this time point was used in subsequent experiments to evaluate the effect of TGF-$\beta_1$. In the concentration-response experiments, the cells were cultured with TGF-$\beta_1$ (0.01, 0.1, 1.0, and 10 ng/ml) for 16 h. A concentration-dependent increase in IL-8 release, which became significant at 1.0 ($P < 0.01$) and 10 ($P < 0.001$; Fig. 1B) ng/ml, was observed.

**Effect of Dex and Act D on TGF-$\beta_1$-induced IL-8 release.** The effect of the corticosteroid Dex and the gene transcription inhibitor Act D on TGF-$\beta_1$-induced IL-8 release was assessed. Pretreatment for 1 h with Act D and Dex (both 1.0 µM) before incubation with TGF-$\beta_1$ (10 ng/ml) for 16 h markedly inhibited TGF-$\beta_1$-induced IL-8 release ($P < 0.001$ for both; Fig. 2).

**Effect of TGF-1β on PGE$_2$ release.** TGF-$\beta_1$ (10 ng/ml) caused a time-dependent accumulation of PGE$_2$ com-
pared with that in the control cells (Fig. 3A). TGF-β1-induced PGE_2 generation was significant after 2 h compared with PGE_2 release from control cells (P < 0.01), and the highest PGE_2 concentration was achieved after 16 h of stimulation (201.7 ± 30.4 pg/ml; P < 0.01). In the concentration response experiments, the cells were cultured with TGF-β1 (0.01, 0.1, 1.0, and 10 ng/ml) for 16 h, and a concentration-dependent increase in PGE_2 release, which was significant at 0.1 (P < 0.05), 1.0 (P < 0.05), and 10 (P < 0.001) ng/ml compared with that in control cells (Fig. 3B), was also observed.

**COX isoform induction in response to TGF-β1.**

COX-2 was undetectable in untreated human ASM cells. In the time-course experiments, COX-2 protein bands appeared 2 h after treatment with TGF-β1 (10 ng/ml), and a strong band was seen at 4, 8, and 16 h, which then diminished at 24 h (Fig. 4). A concentration-dependent increase in COX-2 induction was seen as TGF-β1 concentration was increased from 0.1 to 100 ng/ml (Fig. 4). Pretreatment of the cells with Dex (1.0 μM) abolished the TGF-β1-induced COX-2 expression (Fig. 4). COX-1 was expressed constitutively and did not alter after TGF-β1 treatment (Fig. 4).

**Effect of COX inhibitors on TGF-β1-induced IL-8 and PGE_2 release.** To determine whether COX products played a role in TGF-β1-induced IL-8 release, we pretreated the cells with the nonselective COX inhibitor Indo and the COX-2-selective inhibitor NS-398 for 1 h before incubation with TGF-β1 (10 ng/ml) for 16 h. Neither Indo (10 μM) nor NS-398 (10 μM) had any effect on TGF-β1-induced IL-8 release (Fig. 5A). However, TGF-β1-induced PGE_2 release was significantly inhibited by both Indo (P < 0.01) and NS-398 (P < 0.05; Fig. 5B).

**Cell viability.** Cell viability after 24 h of treatment with all of the chemicals used in this study was consistently >95% compared with vehicle-treated cells (data not shown).

**DISCUSSION**

There are several novel findings in our study. This is the first study to show that TGF-β1 can cause release
of IL-8, induction of COX-2, and generation of PGE\(_2\) by human ASM cells. This is consistent with the hypothesis that human ASM cells can actively modify airway inflammation by expressing and secreting inflammatory products and cytokines (11). Unlike a previous study (24) where prostanoid generation was a prerequisite for bradykinin-induced IL-8 release, COX-2 isoenzyme induction and PGE\(_2\) production were not critical for TGF-\(\beta\)-induced IL-8 release. This suggests that TGF-\(\beta\)1 uses different signaling pathways to release IL-8 compared with those for bradykinin.

We found that human ASM cells released IL-8 in a time- and concentration-dependent manner in response to TGF-\(\beta\)1. However, a significant increase could only be seen after 8 h of treatment, suggesting that the induction of IL-8 is delayed. We have demonstrated that significant IL-8 release from human ASM cells can be observed after 2 h of treatment with bradykinin and that the effect is largely mediated by endogenous prostanoids. The present results suggest that the effect of TGF-\(\beta\)1 may be due to the release of an as yet unidentified mediator, but further work is needed to investigate this. We also showed that the magnitude of IL-8 release by TGF-\(\beta\)1 was, in general, similar to that seen with bradykinin (24). Our findings are similar to the effects of TGF-\(\beta\)1 on IL-8 production in human endometrial stromal cells and human proximal tubular epithelial cells (2, 7). IL-8 has been implicated in several airway diseases such as cystic fibrosis, bronchiectasis, and chronic bronchitis (14). Increased levels of IL-8 have also been demonstrated in bronchoalveolar lavage fluid (37), by macrophages in bronchoalveolar lavage fluid (6), in the blood and bronchial mucosa (33), and by bronchial epithelial cells (17) in asthmatic patients. Overproduction of IL-8 has been implicated in inflammatory cell chemotaxis in asthma, and our results suggest that human ASM cells may respond to TGF-\(\beta\)1 to act as a source of this cytokine in asthma. Previous studies by Pang and Knox (24, 25) with bradykinin and tumor necrosis factor-\(\alpha\) show that they are also able to increase IL-8 release from human ASM cells.

We found that the corticosteroid Dex and Act D, a gene transcription inhibitor, significantly inhibited TGF-\(\beta\)1-induced IL-8 release, suggesting that the increased release of IL-8 was transcriptionally regulated. The efficacy of glucocorticoids in asthma is known to be due to their ability to disrupt cytokine networks in the lung tissue (13). Several studies (reviewed in Ref. 26) showed that there is a nuclear factor (NF)-\(\kappa\)B site on the IL-8 promoter and that Dex is thought to act by inhibiting NF-\(\kappa\)B, thereby suppressing IL-8 transcription.

We also determined the effect of TGF-\(\beta\)1 on COX isoform expression and prostanoid generation from human ASM. We measured PGE\(_2\) as a representative of other prostanoids because Pang and Knox (22) have previously shown that PGE\(_2\) is the dominant prostanoid produced by these cells. Prostanoids are important regulators in the inflammatory process of asthma. Their synthesis is mediated by COX, which exists in two isoforms (18), the constitutive COX-1 and the inducible COX-2. COX-1 is constitutively expressed under physiological conditions in most cells in which it maintains cellular homeostasis by producing physiological levels of prostaglandin (18). Prostaglandins produced by COX-1 generally have a protective function, such as gastric mucus production and renal blood flow maintenance, in non-airway cells. Similarly in the airway, COX-1 is expressed constitutively in ASM cells, mast cells, alveolar macrophages, and fibroblasts (3, 22). In contrast, COX-2 expression is regulated by proinflammatory stimuli including cytokines (15, 22) and proinflammatory mediators such as bradykinin (23). As in previous studies (22, 23), we found that COX-1 was the only isoform expressed under resting conditions, consistent with its putative housekeeping role. We found that TGF-\(\beta\)1 stimulated PGE\(_2\) production through COX-2 induction in a time- and concentration-dependent manner. No previous studies have looked at the effect of TGF-\(\beta\)1 on COX-2 isoform expression in human ASM cells. These findings are similar to the effect of TGF-\(\beta\)1 in astrocytes (15), neurons (15), and lung fibroblasts (9). TGF-\(\beta\)1 may induce
COX-2 by activating the TGF-β response element that is localized in a 166-bp region of the COX-2 gene promoter near the transcriptional initiation site where NF-1 and NF-κB sites are also located (38). TGF-β may also act on other response elements like jun B and serum response factor (38) to induce COX-2. We found that Dex inhibited COX-2 induction. This was likely to be due to inhibition of NF-κB because the human COX-2 promoter region has two NF-κB binding sites (1). Our data on TGF-β1 complements previous studies (18, 22–24) that have shown that COX-2 in ASM is induced by a number of cytokines and proinflammatory stimuli such as bradykinin, suggesting that COX-2 expression may play a pivotal role in the pathophysiological process of asthma.

However, it is unclear whether the increased expression of COX-2 and PGE2 production in human ASM by TGF-β1 would be potentially beneficial or detrimental in the pathogenesis of airway inflammation in asthma. Prostanoids have multiple functions in the airways that include modulating airway tone, cell proliferation, and mucus secretion. PGE2 is an important anti-inflammatory mediator with considerable bronchoprotective effects (27), and the exaggerated PGE2 production as a result of COX-2 induction may be part of a negative feedback mechanism to exert a braking effect on the inflammatory process (13). There are, however, some potential proinflammatory effects of COX-2 induction because PGE2 at high concentrations can cause ASM contraction due to agonism at the thromboxane receptor (12). Induction of COX-2 in inflammatory cells may also exaggerate airway inflammation by producing bronchoconstrictor prostanoids such as PGD2 and PGF2α. Pang et al. (20, 21) have also recently shown that induction of COX-2 in response to IL-1β or bradykinin in human ASM can cause heterologous desensitization of adenylyl cyclase in human ASM, providing a possible explanation for the defective relaxation to β-adrenoceptor agonists demonstrated in human asthmatic bronchi in vitro.

Because our previous studies have shown that prostanoid induction is required for IL-8 release by bradykinin (24), we also studied whether COX-2 induction and PGE2 generation were essential for TGF-β1-induced IL-8 release. In the present study, we used the nonspecific COX inhibitor Indo and the selective COX-2 inhibitor NS-398 to inhibit prostanoid generation. We found that although both Indo and NS-398 markedly inhibited TGF-β1-induced PGE2 generation, they did not inhibit TGF-β1-induced IL-8 release. These data suggest that COX products and COX-2 induction are not directly involved in TGF-β1-induced IL-8 release, unlike bradykinin-induced IL-8 release. This is consistent with results obtained from our laboratory showing that IL-1β-induced IL-8 release is also independent of COX-2 induction and PGE2 generation (Pang and Knox, unpublished data). We have considered possible explanations for these disparate findings. The time course and mechanism of prostanoid generation with bradykinin differs from those with TGF-β1 and IL-1β. Bradykinin causes a much earlier generation of prostaglandins by release of arachidonic acid from phospholipase A2 and subsequent generation of prostanoids from COX-1 as well as from COX-2. It is thus possible that the critical signal in IL-8 generation by bradykinin may be the early release of prostaglandins via COX-1. Because both IL-1β and TGF-β lack the early phase of PGE2 generation, this would explain the lack of prostanoid dependence on IL-8 generation by these agents. Alternatively, bradykinin-induced PGE2 generation may be acting in concert with another signaling pathway that bradykinin but not TGF-β1 or IL-1β activates.

In summary, we have shown that TGF-β1 induces IL-8 release, COX-2 induction, and PGE2 generation from human ASM cells. Our data also suggest that COX-2 induction and prostanoid generation are not essential for TGF-β1-induced IL-8 release. It is likely that these properties of TGF-β1 play a role in the modification of airway inflammation in asthma.

We thank Colin Clelland for providing specimens of human trachea.

L. Pang was supported by the Wellcome Trust.

REFERENCES