Interferon γ Induces Prostaglandin G/H Synthase-2 through an Autocrine Loop via the Epidermal Growth Factor Receptor in Human Bronchial Epithelial Cells

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Abstract

The induction of prostaglandin G/H synthase (PGHS; prostaglandin endoperoxide synthase, cyclooxygenase) by proinflammatory cytokines accounts, at least in part, for the altered eicosanoid biosynthesis in inflammatory diseases. In secondary cultures of normal human bronchial epithelial cells (NHBECs), interferon-γ (IFN-γ, 10 ng/ml for 24 h) increased the amount of prostaglandin E_2 (PGE_2) released in response to stimulation with exogenous arachidonic acid (5 μM). The enhanced production of PGE_2 reflected the upregulation of PGHS-2 as indicated by enhanced expression of PGHS-2 RNA and increased recovery of PGHS-2 protein in NHBECs. IFN-γ did not alter the production of PGE_2 in A549 cells (a human lung adenocarcinoma cell line) or 6-keto-$PGF_{1\alpha}$ in human umbilical vein endothelial cells (HUVECs), although prostaglandin release and/or the expression of PGHS-2 RNA in these cell lines was upregulated by other proinflammatory cytokines. Induction of PGHS-2 RNA in IFN-γ–treated NHBECs, which peaked at 24 h, suggested the presence of an intermediary substance regulating the expression of PGHS-2. When the binding between the epidermal growth factor (EGF) receptor and its ligands was disrupted by a neutralizing antibody (LA-1), IFN-γ failed to upregulate the release of PGE_2 and the expression of PGHS-2 RNA in NHBECs. Furthermore, IFN-γ induced the expression of RNAs for a number of ligands at the EGF receptor TGF-α; heparin-binding EGF-like growth factor (HB-EGF); and amphiregulin in NHBECs, and when administered exogenously, these ligands increased PGE_2 release from NHBECs. Heparin at the concentration that neutralized the function of amphiregulin, or antibodies against TGFα or HB-EGF also reduced the release of PGE_2 from IFN-γ–stimulated NHBECs. These data are consistent with the presence of an autocrine growth factor/EGF receptor loop regulating PGHS-2 expression and PGE_2 synthesis in bronchial epithelial cells. (*J. Clin. Invest.* 1997. 99:1057–1063.) Key words: transforming growth factor-α • heparin-binding EGF-like growth factor • amphiregulin • epithelium • prostaglandin E_2

Introduction

The airway epithelium not only serves as a barrier to isolate the airway microenvironment from external stimuli; it also regulates the function of neighboring cells including bronchial smooth-muscle cells, fibroblasts, mast cells, and inflammatory cells by producing and releasing lipid (prostaglandins, hydroxyeicosatetraenoic acids, lipoxins), gaseous (nitric oxide), and peptide (endothelin, cytokines/chemokines) mediators (1). Among these molecules produced by airway epithelial cells, is prostaglandin E_2 (PGE_2), which regulates bronchial tone as a relaxing factor for smooth-muscle cells (2) and affects the differentiation and function of fibroblasts (3) and mast cells (4). PGE_2 is also an immunomodulator, altering the synthesis of immunoglobulins and cytokines in lymphocytes (5) and macrophages (6). These diverse bioactivities make it important to understand the mechanisms that regulate PGE_2 synthesis in airway epithelial cells.

In these cells, the production of PGE_2 reflects the activity of prostaglandin G/H synthase (PGHS;[1] prostaglandin endoperoxide synthase, cyclooxygenase), which mediates cyclooxygenation and hydroperoxidation of arachidonic acid. Two isoforms of PGHS have been identified; in many systems, PGHS-1 represents most of the constitutive PGHS activity, whereas PGHS-2, which is inducible with tumor promoters, cytokines, growth factors, or lipopolysaccharides is considered the "inflammatory" isoform (7). As we have reported (8), the synthesis of PGE_2 in secondary cultures of normal human bronchial epithelial cells (NHBECs) is regulated differently from prostaglandin synthesis in other cell types. In particular, we have shown that PGHS-2 is the constitutive and dominant isoform in NHBECs. Of three major proinflammatory cytokines (IFN-γ, IL-1β, and TNF-α) with effects on PGE_2 in other cell types (9–15), only IFN-γ induces PGHS-2 RNA expression, protein, and activity in NHBECs. The induction of PGE_2 synthesis is delayed: 12–24 h are required for full PGHS-2 expression. This unusual pattern of induction has led us to reason that IFN-γ induces PGHS-2 in NHBECs by an autocrine growth factor/EGF receptor loop. Our data support the existence of such a loop.

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Received for publication 8 July 1996 and accepted in revised form 12 December 1996.

J. Clin. Invest.

0021-9738/97/03/1057/07 $2.00
Volume 99, Number 5, March 1997, 1057–1063

1. *Abbreviations used in this paper:* HB-EGF, heparin-binding EGF-like growth factor; HUVECs, human umbilical vein endothelial cells; 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; NHBECs, normal human bronchial epithelial cells; PGHS, prostaglandin G/H synthase.

Methods

Materials. Arachidonic acid, heparin, IL-1β, TNF-α, EGF, and TGF-α were obtained from Sigma Chemical Co. (St. Louis, MO). IFN-γ was obtained from GIBCO/BRL Life Technologies (Gaithersburg, MD). Amphiregulin, heparin-binding EGF-like growth factor (HB-EGF), a polyclonal antibody to EGF, and polyclonal antibody to TGF-α were purchased from R&D Systems (Minneapolis, MN). A murine monoclonal antibody to EGF receptor (LA-1), which was raised against a preparation of EGF receptor–rich cell membranes from a human epithelial cell line (A431) as the antigen, was obtained from Genzyme (Cambridge, MA). A polyclonal antibody to HB-EGF (Ab 197) was kindly provided by Deborah Damm and Judy Abraham (Scios-Nova, Mountain View, CA).

Cell culture. NHBECs (Clonetics, San Diego, CA) isolated from the trachea and central airways were maintained in serum-free modified LHC-9 medium (Clonetics) containing EGF (0.5 ng/ml) and bovine pituitary extract (50 μg/ml) as supplements; these cells were used on their second and third passages. Human umbilical-vein endothelial cells (HUVECs), obtained from Clonetics, were cultured in modified MCDB medium with 2% FBS, EGF (10 ng/ml), and bovine brain extract (12 μg/ml). A549 cells were obtained from the American Type Culture Collection (Rockville, MD) and were cultured in F12K medium with 10% FBS. All experiments were performed with confluent cells. 24 h before the experiments reported herein, FBS and growth factors were removed from the culture media for NHBECs and A549 cells; experiments in HUVECs were performed in the presence of 2% FBS but in the absence of EGF and bovine brain extract.

Assay of prostaglandin release. Cells grown to confluence on 24- or 96-well plates were washed twice and then exposed to arachidonic acid (5 μM) in Hanks' balanced salt solution for 30 min at 37°C. The supernatant from each well was immediately mixed with an excess of ice-cold phosphate-buffered saline, and the concentration of PGE_2 (NHBECs or A549 cells) or 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$; for HUVECs) was determined by enzyme-linked immunosorbent assay (Cayman Chemical, Ann Arbor, MI). The concentration of prostaglandins was normalized by the number of viable cells in each well; the latter was determined by the trypan blue exclusion method. The sensitivities (80% B/Bo) of the PGE_2 and the $PGF_{1\alpha}$ assays were 156 and 15.6 pg/ml, respectively.

Reverse transcription PCR and Northern analysis of PGHS transcripts. Total RNA was isolated from freshly harvested cells by the guanidinium thiocyanate-phenol-chloroform extraction method (Stratagene, Inc., La Jolla, CA). Poly(A) RNA was purified by oligo(dT) cellulose chromatography (Pharmacia LKB Biotechnology, Piscataway, NJ). cDNA probes specific to human PGHS-1 and PGHS-2 were synthesized as described previously (8). Probes for EGF, TGF-α, HB-EGF, amphiregulin, and the EGF receptor were generated by reverse-transcription PCR (RT-PCR) using specific oligonucleotide primers (Table 1) and with human lung RNA (Clontech, Palo Alto, CA) as the template. The dideoxynucleotide chain termination method was used to confirm the nucleotide sequences of PCR products before they were used as cDNA probes. Northern analysis was performed as described previously (16). The intensities of bands on autoradiographs were compared by scanning densitometry.

Immunoblot analysis of PGHS proteins. Confluent cells were washed with Hanks' buffered salt solution and lysed in 50 mM Tris buffer (pH 7.6) with 10 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg of pepstatin/ml and 1 μg of leupeptin/ml; protein concentrations were determined with Coomassie blue reagent (Pierce, Rockford, IL). Samples were mixed with equal volumes of loading buffer (100 mM Tris buffer [pH 6.8], 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.05% bromophenol blue) and then subjected to SDS-polyacrylamide gel electrophoresis (4–15% gradient Tris-HCl gel). Sheep PGHS-1, purified from seminal vesicles (Oxford Biomedical Research, Oxford, MI), and sheep PGHS-2, purified from placenta (Oxford Biomedical Research), were loaded on to the gels as controls. The proteins were transferred onto nitrocellulose membranes, which were blocked overnight at 4°C in phosphate-buffered saline/0.05% Tween-20/5% nonfat milk and incubated with primary antibodies (dilutions of 1:250 for antiserum to PGHS-1 and 1:1000 for PGHS-2 [Oxford Biomedical Research]) and a secondary antibody to rabbit IgG 1:10,000 (Amersham, Arlington Heights, IL). Filters were developed with enhanced chemiluminescence reagents (Amersham).

Statistical analysis. The amounts of prostaglandins released from unstimulated and stimulated cells by cytokines or growth factors were compared by means of one-way ANOVA with Bonferroni's correction. The neutralizing effect of LA-1 on growth factor–treated NHBEC was determined by two-way ANOVA. The effects of neutralizing antibodies or heparin were analyzed using Student's *t* test. In all cases a P value of < 0.05 was considered evidence for statistical significance.

Results

Differential upregulation of prostaglandin release by proinflammatory cytokines in NHBECs, A549 cells, and HUVECs. When the capacity of the cytokines, IL-1β (10 U/ml), TNF-α (10 ng/ml) and IFN-γ (10 ng/ml), incubated in culture for 24 h with NHBECs, A549 cells, or HUVECs, to upregulate exogenous arachidonic acid-induced prostaglandin release was examined, distinct differences in response were found. IFN-γ stimulation increased PGE_2 release from NHBECs by $> 500\%$ (2.6 ± 1.0 vs 16.1 ± 5.4 ng $PGE_2/10^5$ cells; mean±SEM, $n = 4$, $P < 0.01$) while the same concentration of IFN-γ had no effect on 6-keto-$PGF_{1\alpha}$ release from HUVECs (47 ± 8 vs 45 ± 5 pg 6-keto-$PGF_{1\alpha}/10^5$ cells; $n = 4$) or on PGE_2 release from A549 cells (0.25 ± 0.07 vs 0.37 ± 0.08 ng $PGE_2/10^5$ cells; $n = 3$) (Fig. 1). In contrast, treatment with IL-1β (10 U/ml), which did not modify PGE_2 release from NHBECs (2.6 ± 1.0 [untreated] vs 3.2 ± 1.1 [IL-1β treated] ng $PGE_2/10^5$ cells; $n = 4$), increased 6-keto-$PGF_{1\alpha}$ release from HUVECs (47 ± 8 vs 390 ± 69 pg 6-keto $PGF_{1\alpha}/10^5$ cells $P < 0.01$, $n = 4$) and PGE_2 release from A549 cells (0.25 ± 0.07 vs 5.25 ± 1.71 ng $PGE_2/10^5$ cells; $P < 0.01$, $n = 3$). Stimulation with TNF-α caused no significant increase in prostaglandin release from NHBECs or A549 cells but did significantly increase the release of 6-keto-$PGF_{1\alpha}$ from HUVECs (47 ± 8 vs 503 ± 34 pg 6-keto $PGF_{1\alpha}/10^5$ cells; $P < 0.05$, $n = 4$). These experiments established that the cytokine response profile of NHBECs, in regard to prostaglandin production, was distinct from that of A549 cells or HUVECs.

Determination of the isoform of PGHS induced in NHBECs by IFN-γ. The isoform of PGHS induced by IFN-γ treatment in NHBECs was determined by Northern blot and immunoblot analysis. As we had previously reported (8), PGHS-2 RNA and protein were constitutively expressed in NHBECs; IFN-γ treatment (10 ng/ml) further upregulated the expression of PGHS-2 RNA and protein in a time-dependent fashion (Fig. 2). The time course of PGHS-2 RNA induction was unusual: there was no significant induction 4 h after IFN-γ treatment, while there was significant induction after 8, 12, or 24 h of cytokine incubation (Fig. 2 *C*). In contrast, IFN-γ did not stimulate PGHS-1 RNA or protein expression in NHBECs studied after 4, 8, 12, or 24 h (data not shown).

Role of an autocrine loop via the EGF receptor for PGHS-2 induction by IFN-γ. The prolonged time required for the upregulation of PGHS-2 in NHBECs by IFN-γ was unexpected since similar to zif/268, nup/475, c-*fos*, and c-*jun*, which are usually induced in less than an hour (17), the PGHS-2 gene is known to be induced with an immediate early time course. Given that the induction of PGHS-2 took > 8 h in NHBECs,

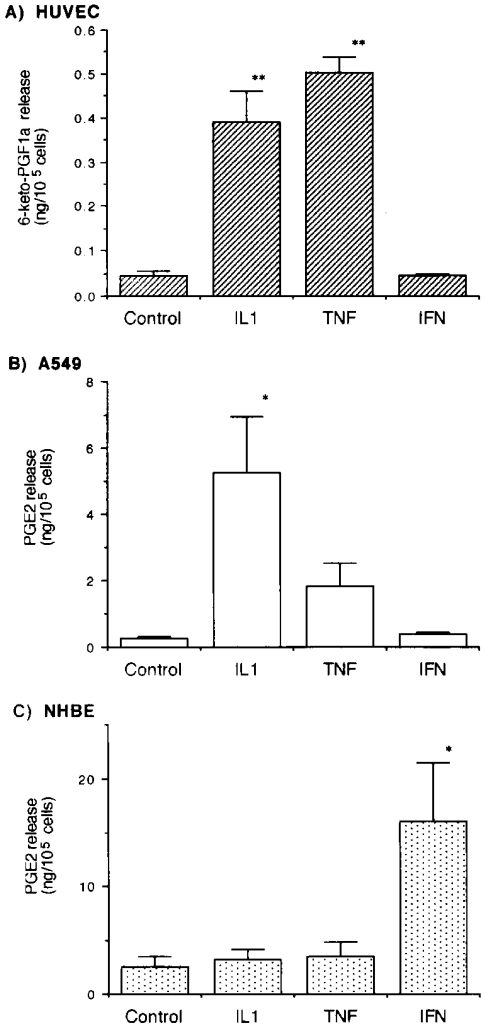

Figure 1. The release of major prostaglandins 6-keto-$PGF_{1\alpha}$ and PGE_2 in response to exogenous arachidonic acid (5 μM, 30 min) from cells preincubated with, interleukin 1β (*IL-1*, 10 U/ml); tumor necrosis factor α (*TNF* 10 ng/ml); or interferon-γ (*IFN* 10 ng/ml) or without cytokines (*control*) for 24 h. Human umbilical vein endothelial cells (*HUVECs*) were used for the study of 6-keto-$PGF_{1\alpha}$ and A549 cells and normal human bronchial epithelial cells (*NHBECs*) were used for the study of PGE_2. Values given are means±SEM (n = 3–4 for each cell type). $^*P < 0.05$ and $^{**}P < 0.01$ vs control group.

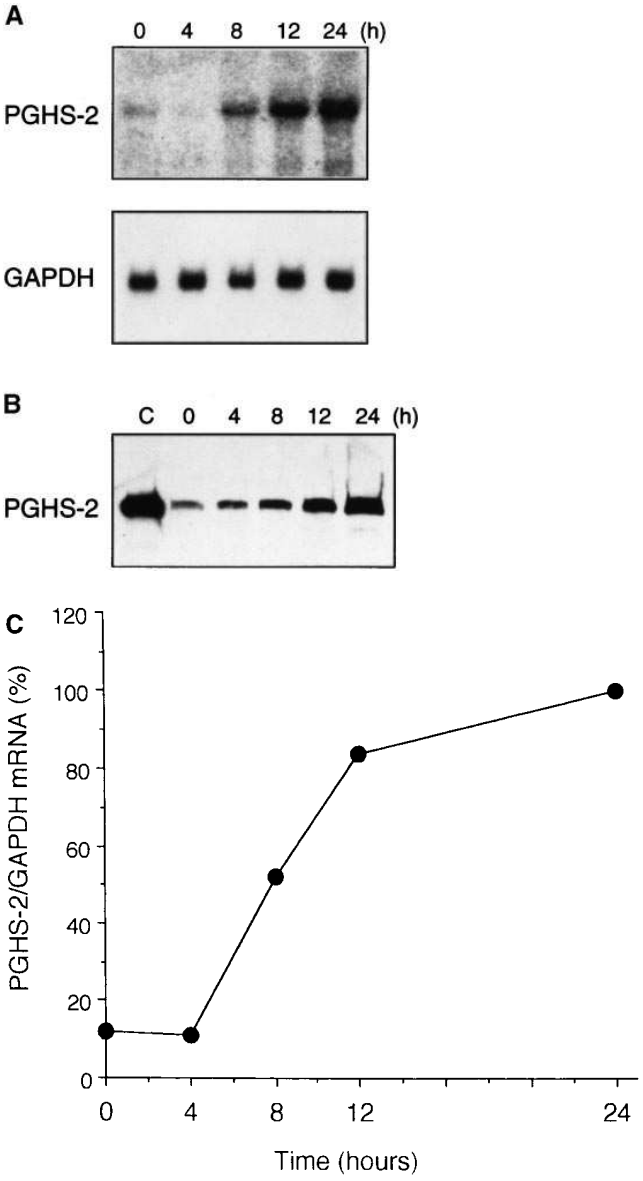

Figure 2. (*A*) Northern analysis of PGHS-2 RNA in NHBECs stimulated with IFN-γ (10 ng/ml) for 0–24 h. (*B*) Immunoblot analysis of PGHS-2 protein in NHBEC stimulated as described in (*A*). Each lane was loaded with 1 μg of sheep PGHS-2 (lane *C*) or 15 μg of cell lysate (0–24 h). (*C*) Relative intensity of signals for PGHS-2/GAPDH RNA in NHBECs treated with IFN-γ (10 ng/ml). The peak intensity for each cell type was designated as 100. Each point represents the mean value of two independent experiments.

we hypothesized the existence of a NHBEC-specific intermediary substance in the signal transduction pathway for IFN-γ. Specifically, since IFN-γ enhances the expression of TGF-α and/or EGF receptors in keratinocytes and mammalian epithelial cells (18, 19) and since EGF induces PGHS-2 in various epithelial cell lines (including the rat tracheal cell line EGV6) (20), we examined the possibility that IFN-γ induced the expression of EGF or related molecules and that stimulation at the EGF receptor upregulated the expression of PGHS-2.

To test this hypothesis, we first ascertained whether IFN-γ could upregulate the expression of the RNA of EGF-related growth factors and the EGF receptor. Three EGF-related growth factors, namely TGF-α, HB-EGF, and amphiregulin, and the EGF receptor were noted by Northern analysis to be constitutively expressed in NHBECs (Fig. 3). In contrast, expression of the RNA for EGF in NHBECs was not detectable by Northern analysis but was evident by RT-PCR (data not shown). IFN-γ treatment (10 ng/ml, 8–24 h) significantly enhanced the expression of the RNA for TGF-α, HB-EGF, and amphiregulin, while no significant change in hybridization signal for EGF RNA or for EGF-receptor RNA was found. Fig. 3 illustrates the induction of the various EGF-related moieties in NHBECs after 12 h of treatment with IFN-γ.

Having shown that NHBECs produced RNA for the growth factors of interest, we next assessed whether EGF and the EGF-related growth factors (TGF-α, HB-EGF, and amphiregulin) from an exogenous source upregulated PGHS-2

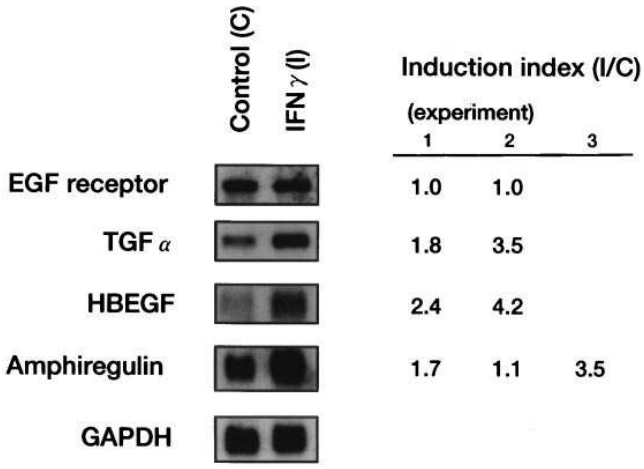

Figure 3. Constitutive (*C*) and IFN-γ–induced (*I*) expression of RNAs of EGF-related growth factors (*TGF-α*, *HB-EGF*, and *amphiregulin*) and the EGF receptor in NHBECs. The intensity of each band was determined by densitometry and normalized by the density of the GADPH RNA signal. Induction was calculated as the ratio of I/C.

release. 4 h after its introduction into cell cultures, each of these ligands significantly upregulated the amount of PGE_2 released from NHBECs in a dose-dependent fashion (Fig. 4). In further studies, we used a monoclonal antibody to the EGF receptor (LA-1; 1 μg/ml) to determine if the upregulation of PGHS-2 activity by EGF-related growth factors was mediated through this receptor. PGE_2 release by exogenous EGF, TGF-α, or amphiregulin was significantly blocked in the presence of LA-1 ($P < 0.01$; Fig. 4). LA-1 did not block PGE_2 release initiated by exogenous HB-EGF in the absence of heparin ($P = 0.052$, Fig. 4). However, coincubation of heparin and LA-1 almost competely neutralized the effect of HB-EGF ($P < 0.001$), data not shown. There was no signficant difference in the number and viability of NHBECs after treatment with the growth factors or LA-1, data not shown.

Once we had established the capacity of LA-1 to block the action of some of the exogenous EGF-related growth factors, we reasoned that, if these factors were produced endogenously by IFN-γ stimulation and if they subsequently acted (in an autocrine fashion) to stimulate EGF receptors, we would be able to block the upreguation of PGHS-2 in NHBECs by IFN-γ by adding LA-1 to cell cultures simultaneously with IFN-γ. Coincubation with LA-1 prevented the upregulated expression of PGHS-2 RNA in IFN-γ–treated NHBECs (Fig. 5 *A*) and inhibited the enhanced release of PGE_2 by 93±4% (Fig. 5 *B*, $P < 0.01$, $n = 8$). Neither IFN-γ alone nor IFN-γ with LA-1 significantly changed the level of PGHS-1 RNA (Fig. 5 *A*). We next reasoned that we could determine which of the EGF ligands was produced in NHBECs by IFN-γ stimulation if we used antibodies to the various proposed intermediates. Thus we examined antibodies to EGF (30 μg/ml), TGF-α (30 μg/ml), and HB-EGF (30 μg/ml) for their capacity to inhibit PGHS activity in NHBECs. We first demonstrated that each antibody inhibited the release of PGE_2 from NHBECs treated with exogenous EGF, TGF-α, or HB-EGF (10 ng/ml) by 75±22, 82±13, and 73±7%, respectively ($n = 4$ for each antibody, $P < 0.05$). Because of the unavailability of a specific neutralizing antibody to amphiregulin, heparin was used as a somewhat selective inhibitor for amphiregulin. At a concentration of 30 μg/ml, heparin inhibited the effect of exogenously administered amphiregulin (500 ng/ml) on PGE_2 release by 90±2% ($n = 8$, $P < 0.05$), but heparin had no significant im-

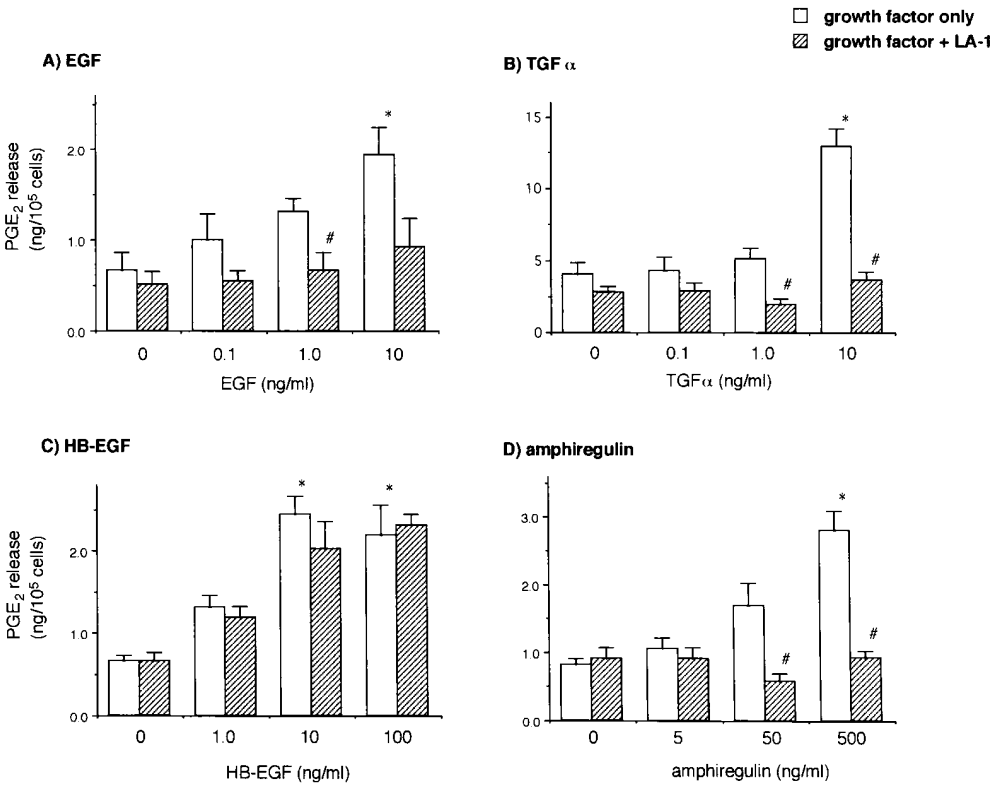

Figure 4. Dose–response relationship between the concentration of EGF-related growth factors (*EGF*, *TGF-α*, *HB-EGF*, and *amphiregulin*) and the amount of PGE_2 released from NHBECs. Cells were treated with EGF, TGF-α, HB-EGF, and amphiregulin for 4 h before the release of PGE_2 was determined as described in Fig. 1. Mean±SEM ($n = 4$). $^*P < 0.05$, compared with the release from unstimulated cells. As indicated the open bars represent the effects of the growth factors alone, while the hatched bars represent the effects of the growth factors when coincubated with LA-1 (1 μg/ml). $^{\#}P < 0.05$, compared with the effects of growth factors alone.

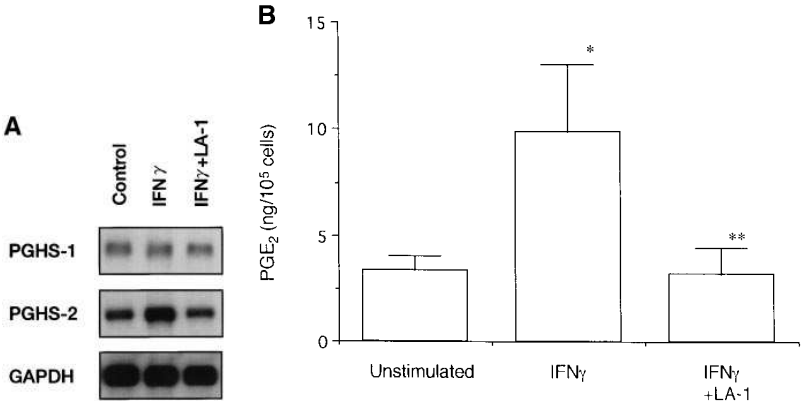

Figure 5. Effects of LA-1 incubation on PGHS RNA (*A*) and PGE_2 production (*B*) in NHBECs. (*A*) The effect of an antibody to EGF receptor (LA-1, 1 μg/ml) on the expression of PGHS-1 and PGHS-2 RNA. These are the representative results from three independent experiments. (*B*) The effects of LA-1 coincubation on the amount of PGE_2 produced by NHBECs when incubated with IFN-γ. There was a significant increase in PGE_2 production induced by IFN-γ; this increase was prevented by pre-incubation with LA-1. $*P < 0.01$, compared with the PGE_2 production from unstimulated cells; $**P < 0.01$, compared with the PGE_2 production from IFN-γ–treated cells.

pact on the enhancement of PGE_2 release by exogenous EGF, TGF-α, and HB-EGF (data not shown). Antibody to TGF-α, antibody to HB-EGF, and heparin each significantly inhibited IFN-γ–induced PGE_2 release from NHBECs by 33±9% ($n = 8$, $P < 0.05$), 27±5% ($n = 6$, $P < 0.05$), and 67±12% ($n = 6$, $P < 0.01$), respectively (Fig. 6). Antibody treatment with an antibody to EGF had no significant effect on IFN-γ–induced PGE_2 release from NHBECs. Unstimulated NHBECs were coincubated with various neutralizing antibodies or heparin for 24 h and the PGE_2 release from these cells was examined. As shown in Fig. 6, antibodies against the EGF receptor (LA-1), TGF-α, or heparin inhibited PGE_2 release significantly, while antibodies against EGF or HB-EGF had no significant effect on PGE_2 release. These data are consistent with the previously noted finding of appreciable levels of constitutive expression of TGF-α, amphiregulin, and EGF receptor RNA (Fig. 3).

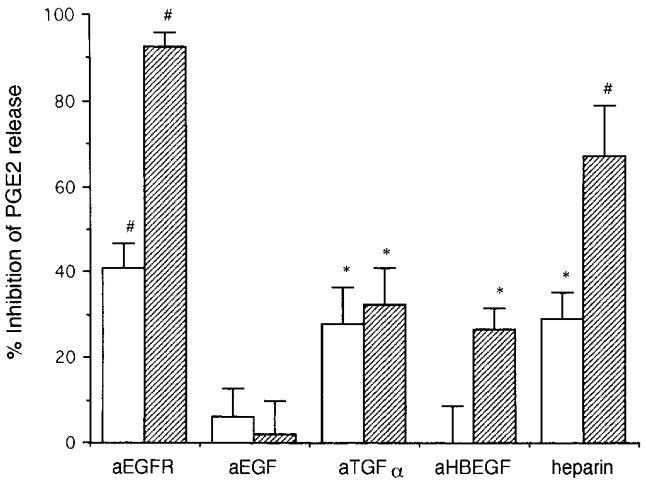

Figure 6. The inhibitory effects of neutralizing antibodies and heparin on the constitutive (*open bars*) and IFN-γ–induced (*hatched bars*) PGE_2 release from NHBEC. Cells were incubated for 24 h with or without IFN-γ (10 ng/ml) in the presence of an antibody to EGF receptor antibody (aEGFR; LA-1, 1 μg/ml, $n = 8$), an antibody to EGF antibody (*aEGF*; 30 μg/ml, $n = 6$), an antibody to TGF-α (*aTGF*; 30 μg/ml, $n = 8$), an antibody to HBEGF (*aHBEGF*; 30 μg/ml, $n = 6$), or heparin (30 μg/ml, $n = 6$) before the release of PGE_2 was determined. Inhibitory effects were calculated by comparison with the amount of PGE_2 released from NHBECs preincubated without neutralizing antibodies or heparin. Mean±SEM. $*P < 0.05$, $^{\#}P < 0.01$, compared with the PGE_2 release from NHBECs preincubated with media alone (*open bars*) or IFN-γ alone (*hatched bars*).

Discussion

The bronchial epithelium is known to modify its phenotype in response to an inflammatory microenvironment. For example, we reported that IFN-γ, IL-1β, and/or TNF-α mediates the upregulation of Type II nitric oxide synthase in airway epithelial cells (including NHBECs) in culture (16). Despite the documented capacity of IL-1β and TNF-α to modify the phenotype of NHBECs and to potently induce PGHS-2 in various cell types (including A549 cells and HUVECs), these cytokines failed to induce gene expression or activity of PGHS-2 in NHBECs. In contrast, IFN-γ, which did not change the amount of prostaglandins released from A549 cells or HUVECs, strongly induced the upregulation of PGHS-2 RNA/protein and the PGHS activity in NHBECs. Our data suggest that IFN-γ exerts its effect on the induction of PGHS-2 in NHBECs through the activation of an autocrine loop involving the EGF receptor expressed on NHBECs and the production (also by NHBECs) of ligands that act at this receptor.

We have previously determined that bronchial smooth-muscle cells and/or U937 cells constitutively express PGHS-1 but not PGHS-2 RNA/protein (8). When these cells are stimulated with PMA, PGHS-2 is the isoform upregulated; in this regard, these two cell types function similarly to vascular endothelial cells, monocytes, and fibroblasts (9, 11–13). We have also shown previously that lung-derived epithelial cells express PGHS isoforms in a pattern that differs markedly from that documented for these two cell types: PGHS-1 RNA or protein is expressed at low levels constitutively; significant amounts of PGHS-2 RNA/protein are constitutively present and are even further upregulated in the presence of PMA.

Because of this unusual pattern of PGHS expression, we examined the mechanisms that regulate induction of PGHS-2 by proinflammatory cytokines in NHBECs. We found that IFN-γ was a strong inducer of PGHS-2 RNA/protein and PGE_2 production in NHBECs while IL-1β or TNF-α had little effect on these cells. In contrast, IL-1β and TNF-α, but not IFN-γ, enhanced the release of prostaglandins in HUVECs and A549 cells. Similar cell-specific gene induction by these cytokines has been documented for the intercellular adhesion molecule-1 (ICAM-1); IL-1β and TNF-α induced the expres-

Table I. Oligonucleotide Primers for RT-PCR

Probe target	Sense primer	Antisense primer
EGF receptor	5′-ACCAGAGTGATGTCTGGAGC-3′	5′-GATGAGGTACTCGTCGGCAT-3′
EGF	5′-GATGGGTACTGCCTCCATGAT-3′	5′-CACTGAGCTAGCTCCATTTGG-3′
TGFα	5′-ACCTGCAGGTTTTTGGTGCAG-3′	5′-ACATGTGGACTCAGACACCAA-3′
HB-EGF	5′-GTGCTGAAGCTCTTTCTGG-3′	5′-ATGCCCAACTTCACTTTCT-3′
Amphiregulin	5′-TCAGTCAGAGTTGAACAGGT-3′	5′-GCTATAGCATGTACATTTCC-3′

sion of this gene in HUVECs, while IFN-γ selectively induced it in NHBECs (21). In the case of ICAM-1 gene expression, the cell specificity of IFN-γ is conveyed by an IFN-γ response element (IRE) containing the sequence TTTCCGGGAAA (22) in the promoter region of its ICAM-1 gene. In the PGHS-2 gene, a C/EBPβ (NF-IL6) consensus sequence and a cyclic AMP response element (CRE) are conserved among species and have been reported to be essential *cis*-acting elements, while none of the *cis*-acting elements responsive to IFN-γ has been described (23–25). Given the absence of elements responsive to IFN-γ in the PGHS-2 gene, we were surprised by the upregulation of PGHS-2 gene expression induced by IFN-γ. On the basis of the slow time course of PGHS-2 induction by IFN-γ–treated NHBECs, we hypothesized the presence of an intermediary substance linking the signal from the IFN-γ receptor to activation of the PGHS-2 gene in NHBECs. Our findings are consistent with an autocrine loop involving the EGF receptor and its ligands as the link between IFN-γ receptor activation and PGHS-2 induction.

A number of lines of evidence are compatible with such a loop. First, we demonstrated the presence of functional EGF receptors on the surface of NHBECs by experiments showing (1) that exogenously administered EGF-related growth factors induced the enhanced release of PGE_2 in NHBECs; (2) that a neutralizing antibody to the EGF receptor significantly decreased the activity of a number of exogenous ligands; and (3) that NHBECs expressed RNA for the EGF receptor. Second, we found that RNAs for the ligands at this receptor, i.e., TGF-α, HB-EGF, and amphiregulin, were expressed in NHBECs and that their expression was upregulated by IFN-γ. Third, we showed that coincubation of NHBECs with an antibody to the EGF receptor prevented the enhancement of PGHS-2 activity by IFN-γ. Fourth, we demonstrated that neutralizing antibodies to EGF ligands or heparin (to "inactivate" amphiregulin) diminished the effects of IFN-γ on the expression of PGHS-2 RNA and enhanced the activity of PGHS (26). In this regard the activity of exogenous HB-EGF was not blocked by LA-1 when used alone but coincubation of LA-1 with heparin almost completely inhibited the release of PGE2 by exogenous HB-EGF. It is possible that when HB-EGF is presented by NHBECs it is in a membrane bound form which is susceptible to inhibition by LA-1 because of the action of glycosaminoglycans in the cellular and extracellular matrix.

Data elsewhere in the literature are consistent with the presence of a similar autocrine loop in airway epithelium in vivo. Rat bronchial epithelium has been shown to constitutively express immunoreactivity for TGF-α and the EGF receptor; this reactivity is further enhanced in bleomycin-induced lung injury (27). Vane and associates (28), who studied a murine air-pouch model of granulomatous inflammation, reported that the induction of PGHS-2 is maximal in the chronic and resolving phase (day 14) rather than in the acute phase (day 3–7) of injury. The slow induction of PGHS-2 activity observed by these investigators is consistent with the late onset of induction of PGHS activity we found in NHBECs. It is interesting to speculate that the loop we have described is even more complex than is now apparent. In specific, our data elucidate a mechanism whereby IFN-γ can upregulate PGHS-2 activity, an event leading to enhanced production of PGE_2 by airway epithelium. It is of interest that PGE_2 selectively suppresses the production of IFN-γ by TH1 cells thereby providing for downregulation of the inflammatory stimulus over a 24–48-h period (5). Whether such an extension of the autocrine loop we have described actually functions and exists is not known.

Our data provide strong evidence for the existence of an autocrine loop regulating PGHS-2 message and activity, however there are a number of issues that bear further consideration. We found that the anti–HB-EGF antibody did not modify PGE_2 release in the absence of IFN-γ stimulation but significantly inhibited PGE_2 release in the presence of IFN-γ stimulation. This finding is consistent with the low constitutive levels of HB-EGF RNA in the unstimulated condition and its subsequent upregulation by IFN-γ. In contrast, when either unstimulated or stimulated NHBECs were incubated with antibodies to TGF-α and heparin there was a decrease in PGE_2 release. These data are consistent with constitutive expression of these ligands at the EGF receptor and their upregulation by IFN-γ. We believe that three ligands, i.e., HB-EGF, TGF-α, and amphiregulin, participate in this autocrine loop and that there is no single predominant ligand. Furthermore, direct measurements of the putative ligands in the culture media is not likely to be informative as it is known that such growth factors may be presented in a juxtacrine fashion, i.e., bound to the NHBECs.

In summary, our data provide evidence consistent with the presence of an autocrine growth factor (TGF-α, HB-EGF, and amphiregulin)/EGF receptor loop that mediates the enhanced expression of PGHS-2 by IFN-γ in NHBECs. Use of LA-1 to inhibit the action of the growth factors induced by IFN-γ at the EGF receptor diminished the effect of IFN-γ on PGE_2 production. Among the airway epithelial cells studied, NHBECs are the only cells to possess a novel mechanism for enhancing PGE_2 production; delineation of this mechanism could lead to the identification of an intervention that would selectively modulate the induction of the PGHS pathway in airway epithelium.

Acknowledgments

This work was supported by National Institutes of Health grant HL19170 and GM47397.

References

1. Jacoby, D.B. 1995. Mediator functions of epithelial cells. *In* Asthma and Rhinitis. W.W. Busse and S.T. Holgate, editors. Blackwell Scientific Publications, Boston. 573–583.

2. Braunstein, G., C. Labat, S. Brunelleschi, J. Benveniste, J. Marsac, and C. Brink. 1988. Evidence that the histamine sensitivity and responsiveness of guinea-pig isolated trachea are modulated by epithelial prostaglandin E_2 production. *Br. J. Pharmacol.* 95:300–308.

3. Bitterman, P.B., M.D. Wewers, S.I. Rennard, S. Adelberg, and R.G. Crystal. 1986. Modulation of alveolar macrophage-driven fibroblast proliferation by alternative macrophage mediators. *J. Clin. Invest.* 77:700–708.

4. Hu, Z.-Q., K. Asano, H. Seki, and T. Shimamura. 1995. An essential role of prostaglandin E on mouse mast cell induction. *J. Immunol.* 155:2134–2142.

5. Phipps, R.P., S.H. Stein, and R.P. Roper. 1991. A new view of prostaglandin E regulation of the immune response. *Immunol. Today.* 12:349–352.

6. Strassmann, G., V. Patil-Koota, F. Finkelman, M. Fong, and T. Kambayashi. 1994. Evidence for the involvement of interleukin 10 in the differential deactivation of murine peritoneal macrophages by prostaglandin E_2. *J. Exp. Med.* 180:2365–2370.

7. Mitchell, J.A., P. Akarasereenont, C. Thiemermann, R.J. Flower, and J.R. Vane. 1994. Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. *Proc. Natl. Acad. Sci. USA.* 90:11693–11697.

8. Asano, K., C.M. Lilly, and J.M. Drazen. 1997. Prostaglandin G/H synthase-2 is the constitutive and dominant isoform in cultured human lung epithelial cells. *Am. J. Physiol.* 271:L126–L131.

9. O'Banion, M.K., V.D. Winn, and D.A. Young. 1992. cDNA cloning and functional activity of a glucocorticoid-regulated inflammatory cyclooxygenase. *Proc. Natl. Acad. Sci. USA.* 89:4888–4892.

10. Jones, D.A., D.P. Carlton, T.M. McIntyre, G.A. Zimmerman, and S.M. Prescott. 1993. Molecular cloning of human prostaglandin endoperoxide synthase type II and demonstration in response to cytokines. *J. Biol. Chem.* 268: 9049–9054.

11. Jackson, B.A., R.H. Goldstein, R. Roy, M. Cozzani, L. Taylor, and P. Polger. 1993. Effects of transforming growth factor β and interleukin-1β on expression of cyclooxygenase 1 and 2 and phospholipase A_2 mRNA in lung fibroblasts and endothelial cells in culture. *Biochem. Biophys. Res. Commun.* 197: 1465–1474.

12. RistimŠki, A., S. Garfinkel, J. Wessendorf, T. Maciag, and T. Hla. 1994. Induction of cyclooxygenase-2 by interleukin-1α. Evidence for post-transcriptional regulation. *J. Biol. Chem.* 269:11769–11775.

13. Crofford, L.J., R.L. Wilder, A.P. RistimŠki, H. Sano, E.F. Remmers, H.P. Epps, and T. Hla. 1994. Cyclooxygenase-1 and -2 expression in rheumatoid synovial tissues. Effects of interleukin-1β, phorbol ester, and corticosteroids. *J. Clin. Invest.* 93:1095–1101.

14. Brum-Fernandes, A.J., S. Laporte, M. Heroux, M. Lora, C. Patry, H.-A. Menard, R. Dumais, and R. Leduc. 1994. Expression of prostaglandin endoperoxide synthase-1 and prostaglandin endoperoxide synthase-2 in human osteoblasts. *Biochem. Biophys. Res. Commun.* 198:955–960.

15. Mitchell, J.A., M.G. Belvisi, P. Akarasereenont, R.A. Robbins, O.J. Kwon, J. Croxtall, P.J. Barnes, and J.R. Vane. 1994. Induction of cyclo-oxygenase-2 by cytokines in human pulmonary epithelial cells: regulation by dexamethasone. *Br. J. Pharmacol.* 113:1008–1014.

16. Asano, K., C.B.E. Chee, B. Gaston, C.M. Lilly, C. Gerard, J.M. Drazen, and J.S. Stamler. 1994. Constitutive and inducible nitric oxide synthase gene expression and activity in human lung epithelial cells. *Proc. Natl. Acad. Sci. USA.* 91:10089–10093.

17. DuBois, R.N., J. Awad, J. Morrow, L.J. Roberts II, and P.R. Bishop. 1994. Regulation of eicosanoid and mitogenesis in rat intestinal epithelial cells by transforming growth factor-α and phorbol ester. *J. Clin. Invest.* 93:493–498.

18. Valyi-Nagy, I., P.J. Jansen, S.M. Albelda, and U. Rodeck. 1992. Cytokine-induced expression of transforming growth factor-α and the epidermal growth factor receptor in neonatal skin explants. *J. Invest. Dermatol.* 99:350–356.

19. Hamburger, A.W., and G.D. Pinnamneni. 1991. Increased epidermal growth factor gene expression by γ-interferon in a human breast carcinoma cell line. *Br. J. Cancer.* 64:64–68.

20. Hamasaki, Y., J. Kitzler, R. Hardman, P. Nettesheim, and T.E. Eling. 1993. Phorbol ester and epidermal growth factor enhance the expression of two inducible prostaglandin H synthase genes in rat tracheal epithelial cells. *Arch. Biochem. Biophys.* 304:226–234.

21. Look, D.C., S.R. Rapp, B.T. Keller, and M.J. Holtzman. 1992. Selective induction of intercellular adhesion molecule-1 by interferon-γ in human airway epithelial cells. *Am. J. Physiol.* 263:L79–L87.

22. Look, D.C., M.R. Pelletier, and M.J. Holtzman. 1994. Selective interaction of a subset of interferon-γ response element-binding proteins with the intercellular adhesion molecule-1 (ICAM-1) gene promoter controls the pattern of expression on epithelial cells. *J. Biol. Chem.* 269:8952–8958.

23. Tazawa, R., X.-M. Xu, K.K. Wu, and L.-H. Wang. 1994. Characterization of the genomic structure, chromosomal location and promoter of human prostaglandin H synthase-2 gene. *Biochem. Biophys. Res. Commun.* 203:190–199.

24. Sirois, J., and J.S. Richards. 1993. Transcriptional regulation of the rat prostaglandin endoperoxide synthase 2 gene in granulosa cells. *J. Biol. Chem.* 268:21931–21938.

25. Xie, W., and H.R. Herschman. 1995. v-*src* induces prostaglandin synthase 2 gene expression by activation of the c-Jun N-terminal kinase and the c-Jun transcription factor. *J. Biol. Chem.* 270:27622–27628.

26. Higashiyama, S., J.A. Abraham, and M. Klagsbrun. 1993. Heparin-binding EGF-like growth factor stimulation of smooth-muscle cell migration: dependance on interactions with cell surface heparan sulfate. *J. Cell Biol.* 122:933–940.

27. Madtes, D.K, H.K. Busby, T.P. Strandjord, and J.G. Clark. 1994. Expression of transforming growth factor-α and epidermal growth factor receptor is increased following bleomycin-induced lung injury in rats. *Am. J. Respir. Cell Mol. Biol.* 11:540–551.

28. Vane, J.R., J.A. Mitchell, I. Appleton, A. Tomlinson, D. Bishop-Bailey, J. Croxtall, and D.A. Willoughby. 1994. Inducible isoforms of cyclooxygenase and nitric-oxide synthase in inflammation. *Proc. Natl. Acad. Sci. USA.* 91:2046–2050.