

Cyclooxygenase-2 Acts as an Endogenous Brake on Endothelin-1 Release by Human Pulmonary Artery Smooth Muscle Cells: Implications for Pulmonary Hypertension

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ABSTRACT

Endothelin-1 is a potent vasoconstrictor and comitogen for vascular smooth muscle. As such, it has been implicated in pulmonary vascular remodeling and in the development of pulmonary hypertension. Prostacyclin has been shown to be an effective therapy for human pulmonary hypertension, reducing morbidity and mortality, although the mechanism of its action is unknown. Here, we show that the combination of TNF- α and IFN- γ induces the release of endothelin-1 from human pulmonary artery smooth muscle cells via increased transcription of prepro endothelin-1. The release of endothelin-1 and the transcription of prepro endothelin-1 mRNA were inhibited by the activity of coincubed cyclooxygenase-2. Endothelin-1 release was also inhibited by a prostacyclin-mimetic (cicaprost). Thus,

under inflammatory conditions, in which vascular smooth muscle is an important source of endothelin-1, the induction of cyclooxygenase-2 represents an endogenous “braking” mechanism. In addition, the beneficial effects of prostacyclin in the treatment of pulmonary hypertension may be caused, at least in part, by the inhibition of endothelin-1 release. Finally, we suggest that these observations may help to explain why patients with pulmonary hypertension experience exacerbations after taking indomethacin and that the newly introduced selective cyclooxygenase-2 inhibitors may increase endothelin-1 production in susceptible patients, leading to vascular remodeling and the development of pulmonary hypertension.

In healthy subjects, pulmonary vascular tone and remodeling is controlled by the balanced, local release of vasoactive mediators, which are chiefly produced by the endothelium, and they act on the underlying smooth muscle. These mediators include the vasodilators nitric oxide and prostacyclin (PGI₂) and the vasoconstrictor endothelin-1 (ET-1). Under inflammatory conditions, these control mechanisms are lost, leading to pulmonary vascular dysfunction, characterized by vasoconstriction and proliferation of vascular smooth muscle (Wort and Evans, 1999). Clinically, this leads to the development of increased pulmonary vascular resistance and the development of pulmonary hypertension. ET-1, a 21-amino acid peptide, is formed from big-ET-1 by the action of membrane-bound metalloproteases termed “endothelin-converting enzymes” (ECEs) (Schmidt et al., 1994). ET-1 is both a

potent vasoconstrictor (Yanagisawa et al., 1988) and a comitogen for vascular smooth muscle (VSM) (Komuro et al., 1988; Bobik et al., 1990), and it has therefore been implicated in the pathogenesis of several, if not all, forms of pulmonary hypertension (Stewart et al., 1991). Although the main source of ET-1 is considered to be the endothelial cell (Yanagisawa et al., 1988), many cell types in vitro can release this peptide when stimulated appropriately. These include VSM cultured from systemic vessels (Hahn et al., 1990; Yu and Davenport, 1995) and, more recently, pulmonary vessels from both animals (Tchekneva et al., 1998, 2000) and humans (Wort et al., 2000, 2001; Upton et al., 2001).

In addition, histological evidence supports a role for the VSM as a producer of ET-1 in disease states associated with pulmonary hypertension. First, in animal models of secondary pulmonary hypertension caused by congestive cardiac failure (Tonnessen et al., 1998), hypoxia (Nakanishi et al., 1999), or chronic air embolus (Tchekneva et al., 1998), pulmonary vascular smooth muscle was shown to synthesize ET-1. Second, in human postmortem studies of pulmonary

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ABBREVIATIONS: TNF- α , tumor necrosis factor α ; IFN- γ , interferon- γ ; PGI₂, prostacyclin; ET-1, endothelin-1; ECE, endothelin-converting enzyme; VSM, vascular smooth muscle; COX, cyclooxygenase; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HPMVE, human pulmonary microvascular endothelial; ELISA, enzyme-linked immunosorbent assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; IL-1 β , interleukin-1 β ; DFU, 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone; ANOVA, analysis of variance.

hypertension, prepro ET-1 mRNA levels were found to be elevated not only in endothelial cells but also in the VSM (Giaid et al., 1993). Finally, we recently demonstrated that ET-1 release from stimulated human pulmonary artery smooth muscle cells *in vitro* promotes cellular proliferation, confirming an important autocrine role for this mediator in vascular remodeling (Wort et al., 2001).

PGI₂ is an important therapy for both primary and secondary forms of pulmonary hypertension (Gaine and Rubin, 1998), and its prolonged administration is associated with a marked improvement in morbidity and survival (Barst et al., 1994; McLaughlin et al., 1998). The benefits of PGI₂ in the treatment of primary pulmonary hypertension outweigh its vasodilator effects. Thus, the slow and progressive reduction in pulmonary vascular resistance suggests a reversal of the remodeling processes (McLaughlin et al., 1998), although the underlying mechanisms involved remain unclear.

We have shown recently that human VSM cells, including those from the pulmonary circulation, can be stimulated to release PGI₂ after the induction of cyclooxygenase-2 (COX-2) (Bishop-Bailey et al., 1998; Jourdan et al., 1999a,b). Furthermore, we have shown that both endogenously released PGI₂, via COX-2 induction, and an exogenous PGI₂ mimetic, cicaprost, suppress proliferation of human pulmonary artery smooth muscle (Jourdan et al., 1999b).

The aim of this study was to investigate the effects of exogenous and endogenous PGI₂ on ET-1 release by human pulmonary artery smooth muscle cells. Such observations may have important implications for the understanding of the mechanism of action of PGI₂ in the treatment of pulmonary hypertension and may help to direct future therapy. Also, with the recent introduction of new drugs that selectively inhibit COX-2, our observations may have important implications for patients with pulmonary hypertension who are taking these drugs for other conditions (e.g., rheumatoid arthritis).

Materials and Methods

Cell Culture. Specimens of human pulmonary artery from healthy segments of lung were obtained from patients undergoing pulmonary resection at the Royal Brompton Hospital (London, UK). Under sterile conditions, main pulmonary vessels were dissected clean from adventitia and opened longitudinally. The endothelium was removed mechanically with a scalpel blade, and the vessel was cut into 3- to 4-mm² pieces. Dulbecco's modified Eagle's medium (DMEM) was added to the tissue in a tissue-culture flask supplemented with 15% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin, 2.5 µg/ml amphotericin B, and nonessential amino acids (L-alanine, L-asparagine, L-aspartate, L-glutamate, glycine, L-proline, and L-serine, at the manufacturer's recommended concentrations; Invitrogen, Carlsbad, CA). Tissue was incubated at 37°C in an atmosphere of 5% CO₂ and 95% air, and after approximately 4 to 6 weeks, vascular smooth muscle cells began to explant and colonize the flasks. Cells were confluent after approximately 4 weeks. Human pulmonary artery smooth muscle cells were identified by characteristic "hill-and-valley" morphology and, for representative cultures, confirmed by staining with fluorescein isothiocyanate-labeled anti-smooth muscle α -actin antibody. Cells between passage 2 and 9 were seeded onto 96-well plates. In some experiments, human pulmonary microvascular endothelial (HPMVE) cells were used as a comparison. These cells were purchased from BioWhittaker (Wokingham, UK). HPMVE cells were maintained in endothelial cell growth medium 2-micro-

vascular (BioWhittaker) supplemented with growth factors, as described previously (Blease et al., 1999).

Measurement of Endothelin-1 Release. To assess the effect of cytokines and drugs on ET-1 release, cells were seeded onto 96-well culture plates, were allowed to grow to confluence (10,000 cells/well), were serum-deprived for 24 h (DMEM as described above without FCS and with 0.1% w/v bovine serum albumin), and then were incubated in DMEM as described above, including 10% FCS with or without the addition of cytokines or drugs for another 24 h. The supernatant was then removed, and the concentration of ET-1 was measured by using a commercially available sandwich ELISA (R & D Systems, Abingdon, UK), either immediately or after storage at -80°C. All release experiments were carried out in the presence of the peptidase inhibitors captopril (1 µM), bestatin (1 µM), thiorphan (1 µM), and bacitracin (3 µg/ml) to prevent the degradation of ET-1 by endogenous peptidases (Woods et al., 1999). Cell viability was assessed in representative experiments by measuring the ability of cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan, a measure of cell respiration. At the end of the experiment, after supernatant removal, cells were incubated with MTT (1 mg/ml) for 15 min at 37°C. After removal of MTT, the cells were made soluble in 100 µl of dimethyl sulfoxide. Formazan levels were assessed by the measurement of optical density at 550 nm.

RNA Extraction and Polymerase Chain Reaction (PCR). To investigate alterations in the levels of prepro ET-1, ECE-1, and COX-2 mRNA, RT-PCR was performed. Human pulmonary artery smooth muscle cells were cultured in 6-well plates for 1 to 48 h with or without FCS and combinations of cytokines. Total RNA was extracted according to the method described by Chomczynski and Sacchi (1987) with minor modifications. Total RNA (0.6 µg) was converted to cDNA using reverse-transcriptase (Promega, Southampton, UK). PCR was performed using primers selected for prepro ET-1, ECE-1b/c, COX-2, and β -actin from sequences published in GenBank using a Crocodile III thermocycler (Applied Biosystems, Chester-le-Street, UK). Annealing temperatures and cycle numbers were 53°C and 25 cycles, 54°C and 28 cycles, and 57.5°C and 19 cycles for prepro ET-1, ECE-1b/c, and β -actin, respectively. Sequences used were the following: sense for prepro ET-1, 5'-GATGCCAATGTGCTAGCCAA-3'; antisense for prepro ET-1, 5'-CTGATGGAAGCCAGTGAAGA-3'; sense for ECE-1b/c, 5'-GATGTCGACGTACAAGC-3'; antisense for ECE-1b/c, 5'-CTGTTGGAGTCTTGGAATC-3'; sense for β -actin, 5'-GGCACCACACCTTCTACAATG-3'; antisense for β -actin, 5'-CAGGAAGGAAGGTTGGAAGAG-3'; sense for COX-2, 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3'; and antisense for COX-2, 5'-AGATCATCTCTGCCTGAGTATCTT-3'. PCR products were analyzed on 1% agarose gels and bands analyzed for densitometry.

Western Blotting. Confluent monolayers of human pulmonary artery smooth muscle cells were grown on 6-well plates. After serum deprivation for 24 h, cells were treated with serum alone or with a combination of cytokines for 24 h. After washing the cells twice with ice-cold phosphate-buffered saline, whole-cell extracts were prepared by scraping the cells in ice-cold phosphate-buffered saline containing 0.1% Triton X-100, 10 mM EDTA, 2 µM leupeptin, 1 µM pepstatin, and 1 mM phenylmethylsulfonyl fluoride.

Protein concentrations were estimated by use of a Bio-Rad protein assay (Bio-Rad, Hemel Hempstead, UK). Protein extracts were boiled for 5 min in sample loading buffer. Protein (25 µg) was loaded for each condition onto a 10% acrylamide gradient gel and subjected to electrophoresis for 1 h at 100 V. The separated proteins were electrotransferred to nitrocellulose (Hybond C; Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) at 100 V for 1 h, and the blots were incubated in blocking buffer (0.1% Tween 20 in Tris-buffered saline with 5% low-fat milk) overnight at 4°C on a rocking platform. Blots were then incubated with primary antibody against COX-2 protein (1/1000 dilution; Cayman Chemical, Ann Arbor, MI) for 1 h in blocking buffer at room temperature, and after washing for 3 × 5 min, the blots were exposed to the secondary antibody and then conjugated to horseradish peroxidase (1/4000 dilution; DAKO, Bucks, UK) for 1 h in blocking buffer at room

temperature. Protein bands were visualized by using enhanced chemiluminescence reagent (Amersham Biosciences) and by exposure to high-performance enhanced chemiluminescence film (Hyperfilm ECL; Amersham Biosciences).

Materials. DMEM, penicillin, streptomycin, glutamine, nonessential amino acids, sodium pyruvate, and FCS were obtained from Invitrogen (Carlsbad, CA). The cytokines TNF- α , IFN- γ , and IL-1 β as well as the ET-1 ELISA were obtained from R & D Systems (Abingdon, UK). DFU was a gift from Dr. Ford-Harrison at Merck Eurolab Ltd. (Poole, Dorset, UK). Indomethacin, thiorphan, captopril, rolipram, dibutyl-cyclic AMP, forskolin, MTT, and bestatin were all obtained from Sigma Chemical Co. (Poole, Dorset, UK). Primers for PCR were constructed by Invitrogen. Cicaprost was a kind gift from Schering AG (Berlin, Germany).

Statistical Analysis. All data are reported as means \pm S.E.M. from n experiments. Statistical analysis was conducted with the use of one-way analysis of variance (ANOVA) with Bonferroni post-test analysis. P values less than or equal to 0.05 were considered significant.

Results

IL-1 β Inhibits ET-1 Release from Human Pulmonary Artery Smooth Muscle Cells. Human pulmonary artery smooth muscle cells released detectable levels of ET-1 into the supernatant, which were significantly increased in the presence of a combination of the cytokines TNF- α and IFN- γ , both at 10 ng/ml for 24 h (8.9 ± 3.8 versus 24.1 ± 5.3 pg/ml, $p < 0.01$, $n = 8$) (Fig. 1). Basal levels of ET-1 release were not affected by either cytokine alone (data not shown). Inclusion of IL-1 β (10 ng/ml) into the cytokine mixture of TNF- α plus IFN- γ resulted in a significant inhibition of ET-1 release (24.1 ± 5.3 versus 7.1 ± 2.4 pg/ml, $p < 0.05$, $n = 8$; Fig. 1A). As with TNF- α or IFN- γ , IL-1 β alone had no significant effect on ET-1 release by these cells (8.9 ± 3.8 versus 3.3 ± 2.0 pg/ml). None of the treatments affected cell viability, as determined by MTT assay (data not shown).

IL-1 β Stimulates ET-1 Release from Human HPMVE Cells. Stimulation of HPMVE cells with TNF- α or IL-1 β alone increased ET-1 release into the supernatant, whereas

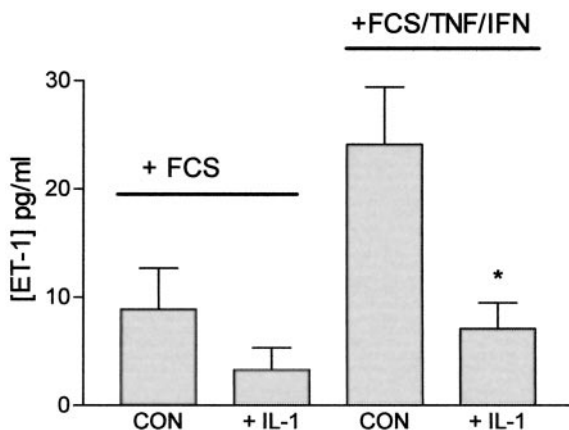


Fig. 1. The effect of cytokines on the release of ET-1 from human pulmonary artery smooth muscle cells. Human pulmonary artery smooth muscle cells were cultured for 24 h in the presence of various combinations of the cytokines TNF- α , IFN- γ , and IL-1 β (10 ng/ml each). ET-1 released into the supernatant was measured by ELISA. Data shown are means \pm S.E.M. from eight patients' cells measured in triplicate. The addition of IL-1 β significantly inhibited release of ET-1 stimulated by TNF- α and IFN- γ . *, $P < 0.05$ by one-way ANOVA. This effect was not seen in human pulmonary microvascular endothelial cells (Table 1). CON, control.

IFN- α had no detectable effect. Furthermore, no "synergy" was seen when cells were treated with TNF- α plus IFN- γ . Finally, IL-1 β had no additional effect on ET-1 release from cells treated with TNF- α and IFN- γ (Table 1).

Coincubation with IL-1 β Inhibits Prepro ET-1 mRNA Transcription Induced by TNF- α and IFN- γ . Incubation of human pulmonary artery smooth muscle cells with TNF- α plus IFN- γ (10 ng/ml each) led to an increase in expression of prepro ET-1 mRNA over a 48-h period, as detected by semi-quantitative RT-PCR. A much smaller increase was seen with serum alone, consistent with our previous findings (Wort et al., 2001). The increase with TNF- α plus IFN- γ was inhibited by the inclusion of IL-1 β (also at 10 ng/ml; Fig. 2, A and B). There were no changes seen in the level of ECE-1 (data not shown) or β -actin (Fig. 2, A and B) mRNA under these conditions.

Inhibitory Effect of IL-1 β on ET-1 Release from Human Pulmonary Artery Smooth Muscle Cells Is Dependent on COX-2 Activity. Both the nonspecific COX inhibitor indomethacin (10 μ M) and the selective COX-2 inhibitor DFU (1 μ M) reversed the inhibition in ET-1 release caused by IL-1 in human pulmonary artery smooth muscle cells stimulated with TNF- α and IFN- γ for 24 h (Fig. 3, A and B). We have shown previously that these cytokines stimulate the release of PGI $_2$ (measured by the formation of 6-keto PGF $_{1\alpha}$) via a COX-2-dependent pathway (Jourdan et al., 1999a,b). Neither indomethacin nor DFU had a significant effect on ET-1 release from cells under basal culture conditions, or when treated with IL-1 β alone or with the combination of TNF- α and IFN- γ without IL-1 β (data not shown). The vehicle for both drugs was 0.01% dimethyl sulfoxide, which had no effect on ET-1 release.

Effect of Cytokines on COX-2 Protein Expression in Human Pulmonary Artery Smooth Muscle Cells. In human pulmonary artery smooth muscle cells deprived of serum for 24 h, there was no COX-2 protein as detected by Western blot analysis. However, these cells expressed COX-2 protein when stimulated separately with IL-1 β but not with TNF- α and IFN- γ (10 ng/ml each) for 24 h. When IL-1 β was added together with TNF- α and IFN- γ , COX-2 expression was further increased in these cells (Fig. 4, A and B). Levels of COX-2 protein expression were reflected in levels of COX-2 mRNA. Thus, IL-1 β alone caused a small increase in COX-2 mRNA level during basal conditions which was sustained (data not shown), whereas TNF- α and IFN- γ increased expression at 4 h after stimulation, an effect that declined by 24 and 48 h. However, the addition of IL-1 β to the combination of TNF- α and IFN- γ resulted in a large and sustained in-

TABLE 1

The effect of cytokines on release of ET-1 from human pulmonary microvascular endothelial cells
Cells were incubated with various combinations of the cytokines (10 ng/ml each) for 24 h. Data are expressed as mean \pm S.E.M. for six wells from two cell lines.

Cytokine	ET-1 Release pg/ml
Control	256 ± 15
TNF	$399 \pm 21^*$
IFN	205 ± 28
IL-1	$370 \pm 13^*$
TNF/IFN	$340 \pm 8^*$
TNF/IFN/IL-1	$322 \pm 12^*$

*, Statistical differences where $P < 0.05$ (calculated using one-way ANOVA).

crease in the COX-2 mRNA level (Fig. 4C). There were no changes seen in the levels of β -actin at any of the above-described conditions.

Inhibitory Effect of Cicaprost and cAMP-Increasing Agents on ET-1 Release from Human Pulmonary Artery Smooth Muscle Cells. Cicaprost (10^{-9} to 10^{-6} M), a PGI₂ mimetic, caused concentration-dependent inhibition of ET-1 release from human pulmonary artery smooth muscle cells treated with TNF- α and IFN- γ ($n = 8$; Fig. 5A). To investigate whether this inhibition was a cyclic AMP-mediated effect, agents that increase intracellular cyclic AMP were incubated with cytokine-stimulated cells. Forskolin (10 μ M), an activator of adenylate cyclase; dibutyryl¹⁵-cyclic AMP (100 μ M), a stable analog of cyclic AMP; and rolipram (100 μ M), a type IV phosphodiesterase inhibitor, all resulted in significant inhibition of TNF- α -plus-IFN- γ -stimulated ET-1 release (Fig. 5B). None of the treatments affected cell viability, as determined by MTT assay (data not shown).

Discussion

Vascular smooth muscle has an important role in the generation of inflammatory mediators in diseases of the systemic circulation (Woods et al., 1999). We (Wort et al., 2001; Jourdan et al., 1997, 1999a,b), and others (Upton et al., 2001) have suggested a similar role for vascular smooth muscle in the pulmonary circulation in diseases leading to an increase in pulmonary vascular resistance. We showed previously that human pulmonary vascular smooth muscle cells can be stimulated to synthesize ET-1 and, in a separate study, release prostacyclin after the induction of COX-2. In the current study, we show that the release of ET-1 by these cells is

limited when COX-2 is induced simultaneously. We suggest that these observations have important implications clinically and may help to explain why patients with pulmonary hypertension experience exacerbations after taking indomethacin.

Traditionally, endothelial cells derived from either systemic or pulmonary vessels were considered to be the principal source of ET-1 in the vasculature (Yanagisawa et al., 1988). However, studies have now emerged from our own group (Wort et al., 2001) and from others (Upton et al., 2001) that identify the pulmonary vascular smooth muscle as an important source of this peptide. Thus, we have shown previously that these cells release ET-1 under standard control culture conditions, which mediates proliferative responses in an autocrine manner (Wort et al., 2001). Pulmonary hypertension can be considered to be an inflammatory condition and as such is associated with increased levels of cytokines (Tuder and Voelkel, 1998). In the current study, the basal release of ET-1 we reported previously (Wort et al., 2001) was significantly increased by the combination of TNF- α plus INF- γ . In accordance with our data regarding mature peptide release, we also found that mRNA levels for prepro ET-1 were elevated by TNF- α plus INF- γ , which establishes that the cytokines are acting either at the transcriptional level or by inhibiting mRNA degradation. Neither of these two cytokines influenced ET-1 release from our cells when given

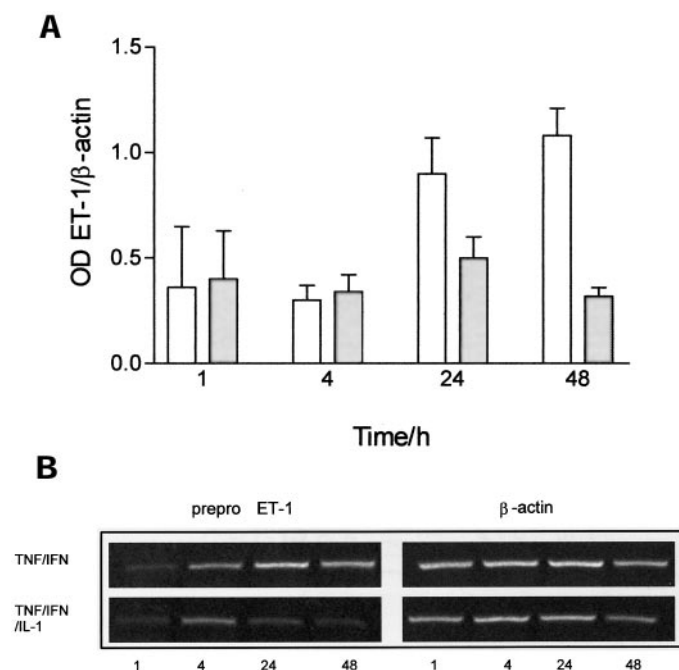


Fig. 2. RT-PCR analysis of prepro ET-1 mRNA after stimulation with cytokines. A representative gel showing the time course for prepro ET-1 mRNA after stimulation with TNF- α and IFN- γ , with and without the addition of IL-1 β . A comparison is made with mRNA for human β -actin at the same time points. A, data from three patients' cells, represented as means \pm S.E.M., for prepro ET-1/ β -actin optical density (OD) ratios (□, TNF- α plus IFN- γ ; ■, addition of IL-1 β). B, a representative gel.

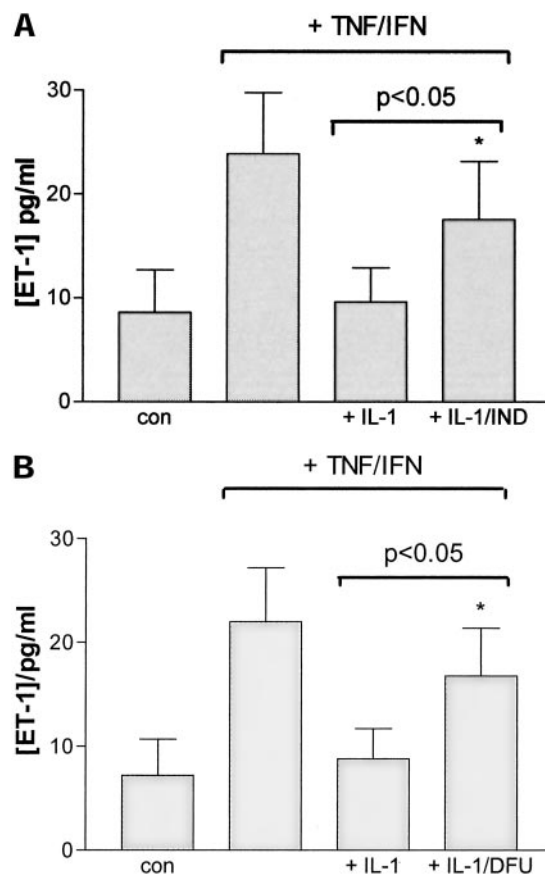


Fig. 3. The effect of the nonselective COX inhibitor indomethacin (IND) (10 μ M) (A) and the selective COX-2 inhibitor DFU (1 μ M) (B) on ET-1 release on cells treated with TNF- α , IFN- γ , and IL-1 β . Both agents significantly reversed the inhibition observed with IL-1 β . *, $P < 0.05$ by one-way ANOVA; $n = 6$ patients' cells for DFU experiments and 5 patients' cells for the indomethacin experiments. CON, control.

alone, revealing a clear synergy between TNF- α and IFN- γ in this response. Although the mechanisms mediating the synergistic actions of TNF- α and IFN- γ on ET-1 release are unknown, several other genes, such as IL-6 and vascular cell adhesion molecule 1, are also regulated in this way (Paludan, 2000), possibly via interactions of the nuclear factor- κ B/p65 and interferon regulatory factor-1 pathways. Whether a similar phenomenon is responsible for the synergistic release of ET-1 by TNF- α and IFN- γ and the exact nature of the requirement this may have on the presence of serum in our cells are not central to the current study, but they remain a matter of investigation.

Like TNF- α , IL-1 β is implicated in the pathogenesis of pulmonary hypertension, in which its elevated levels correlate with disease severity (Humbert et al., 1995). As with the addition of TNF- α and IFN- γ separately, IL-1 β did not stimulate the release of ET-1 from vascular smooth muscle cells. These observations are in direct contrast to those that we

obtained using human pulmonary endothelial cells, in which IL-1 β , like the other cytokines, induced ET-1 release. Indeed, IL-1 β actually tended to reduce the basal release of ET-1 from our vascular smooth muscle cells, although this effect did not reach statistical significance. Moreover, IL-1 β dramatically reduced the ability of TNF- α plus IFN- γ to release ET-1. A similar inhibitory relationship between IL-1 β and TNF- α plus IFN- γ was seen on ET-1 mRNA levels. IL-1 β is not normally reported as an inhibitor of inflammatory gene expression, and it is not obvious how its signaling pathway may directly inhibit that of TNF- α and IFN- γ . Thus its inhibitory effect may be indirect and caused by the induction of a second gene product.

Using human pulmonary artery smooth muscle cells, we have shown previously that IL-1 β induces the expression of COX-2 accompanied by the release of PGI₂ and PGE₂ (Jourdan et al., 1999a,b). Thus, COX-2 products could mediate the inhibitory effect of IL-1 β on ET-1 release. In support of this concept, we found that COX-2 mRNA and protein were induced by IL-1 β in cells costimulated to release ET-1 with

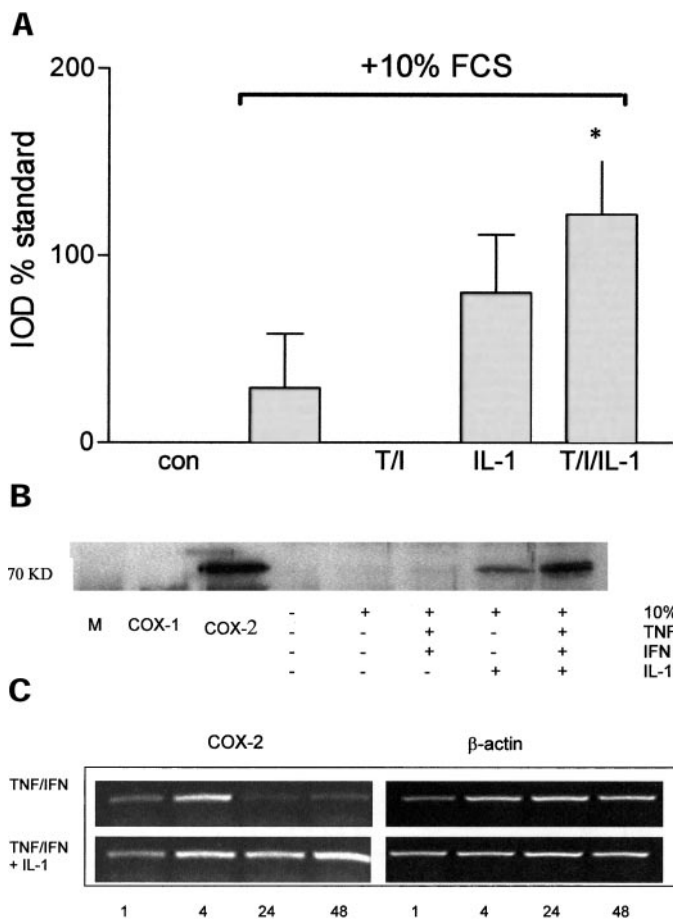


Fig. 4. A, the effect of cytokines and 10% FCS on COX-2 protein and mRNA as determined by Western blot analysis and RT-PCR, respectively. The combination of TNF- α (T), IFN- γ (I), and IL-1 β (IL-1) is required for maximal COX-2 protein expression (data from three patients' cells and expressed as means \pm S.E.M.). *, $P < 0.05$; statistically significant difference (tested by one-way ANOVA) between cytokine treatment versus serum alone; con, control. B, a representative Western blot in which M represents the markers of molecular mass, COX-1 and COX-2 are lanes with the corresponding control peptide, and 10% is 10% FCS. The band shown corresponds to a molecular mass of approximately 70 kDa. Similarly, a combination of all three cytokines is required for maximal COX-2 mRNA expression, as observed over a 48-h time course (C, representative blot, with two additional patients' cells showing identical results). A comparison is made with mRNA for human β -actin.

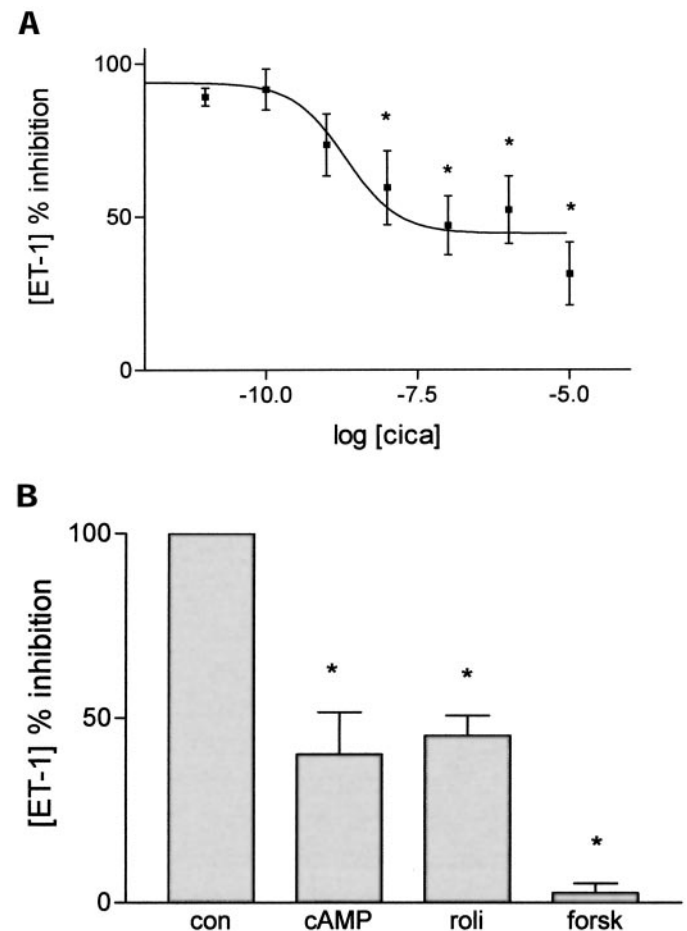


Fig. 5. A, the effect of increasing concentrations of the prostacyclin-mimetic cicaprost on ET-1 release from human pulmonary artery smooth muscle treated with TNF- α and IFN- γ in the presence of 10% FCS. Data shown are means \pm S.E.M. from six patients' cells, repeated in triplicate. *, $P < 0.05$ by one-way ANOVA. B, the effect of agents that increase intracellular cyclic AMP on the release of ET-1 from human pulmonary artery smooth muscle cells treated with TNF- α and IFN- γ for 24 h in the presence of 10% FCS. Forskolin, an adenylate cyclase activator, dibutyryl cyclic AMP, and rolipram, a phosphodiesterase inhibitor, all significantly inhibited ET-1 release. *, $P < 0.05$, $n = 5$ patients' cells; con, control; rol, rolipram; forsk, forskolin.

TNF- α plus INF- γ . In addition, the inhibitory effects of IL-1 β were reversed when COX-2 activity was blocked using either indomethacin or DFU. Our results using primary human pulmonary smooth muscle cells are supported by one other study in the literature in which IL-1 β inhibited ET-1 release by alveolar epithelial cells (Odoux et al., 1997) via a COX-dependent pathway. Moreover, in the only clinical study to our knowledge to address the effect of COX inhibition on pulmonary hemodynamics in patients with pulmonary hypertension, administration of indomethacin resulted in increased pulmonary vascular resistance (Hermiller et al., 1982).

Nonsteroidal anti-inflammatory drugs including indomethacin have been reported to have effects independent of COX inhibition (Weissmann et al., 1987), which may explain the data we report here on ET-1 production. However, we were able to mimic the inhibitory effects of endogenous COX-2 activity on ET-1 production by the addition of a PGI₂ mimetic (cicaprost). In contrast to the observation with cicaprost, exogenous PGE₂ had a very limited effect on ET-1 release from our cells (S. Wort, T. Evans, J. Mitchell, unpublished observations). This finding strongly suggests an action of both endogenous COX-2 products and exogenous cicaprost on prostanoid IP receptors. Similar results have been obtained in bovine endothelial cells stimulated with serum and treated with PGI₂, in which inhibition seemed to occur at both transcriptional and post-translational levels (Prins et al., 1994; Razandi et al., 1996).

Prostanoid IP receptor activation results in stimulation of adenylate cyclase followed by a subsequent increase in cyclic AMP. In addition to PGI₂, we were able to inhibit ET-1 production by incubating smooth muscle cells with several other cyclic AMP-elevating agents. Specifically, rolipram, which inhibits type IV phosphodiesterase enzymes, forskolin, which activates adenylate cyclase, and dibutyryl cyclic AMP, which is a mimic of intracellular cyclic AMP, all decreased ET-1 production. The putative cyclic AMP responsive element-binding protein recognition site is on the prepro ET-1 promoter (Wingender et al., 2001), and this may well be the site at which the inhibitory effects on ET-1 production described here occurs.

These findings have raised important clinical implications. First, the beneficial effects of PGI₂ in the treatment of pulmonary hypertension may now be explained, at least in part, by its actions on ET-1 release. This is supported not only by our data, but also in a clinical study in which intravenous PGI₂ was shown to have a direct effect on ET-1 levels, suggesting reduced synthesis in the lungs (Langleben et al., 1999). Second, as endogenous PGI₂ production is reduced in patients with primary pulmonary hypertension (Christman et al., 1992), the coexisting increased levels of ET-1 may be secondary to the loss of an endogenous "brake" on ET-1 release. Because patients with primary pulmonary hypertension are generally treated with the anticoagulant warfarin, the coadministration of standard nonselective COX-1/COX-2 nonsteroidal anti-inflammatory drugs, which increase bleeding times by inhibiting COX-1, is clinically contraindicated. With the development and availability of selective COX-2 inhibitors, which do not affect platelet function, clinicians may assume a better profile in pulmonary hypertension. However, we predict that the use of COX-2 selective inhibitors may actually increase endogenous ET-1 release in such

patients and thereby exacerbate or even initiate vascular remodeling and pulmonary hypertension. Finally, increasing the capacity of pulmonary cells to form PGI₂ endogenously in vivo may be an additional/better therapy for pulmonary hypertension than the current system of continuous PGI₂ intravenous infusion. Certainly, animal models would suggest this to be the case. Thus, transfection of PGI₂ synthase cDNA into rat VSM cells resulted in increased PGI₂ synthesis and inhibition of cell growth (Hara et al., 1995). Moreover, overexpression of PGI₂ synthase in transgenic mice protected against the development of hypoxic pulmonary hypertension (Geraci et al., 1999).

In summary, we have shown that the induction of COX-2 in human pulmonary artery smooth muscle cells limits endogenous ET-1 release from these cells. This effect is similar to that seen with the addition of exogenous PGI₂ and other cyclic AMP-increasing agents. This has important implications both for understanding the pathogenesis of human pulmonary hypertension and in directing future therapies.

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