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Regulation of the inducible cyclo-oxygenase pathway in human cultured airway epithelial (A549) cells by nitric oxide

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1 In airway epithelium, nitric oxide (NO) is synthesized in the setting of inflammation by inducible nitric oxide synthase (iNOS). Although the role of epithelial derived NO in the regulation of human airways is unknown, prostaglandin E_2 (PGE_2) is recognised as an important inhibitory mediator in human airways. Cyclo-oxygenase (COX) is the rate limiting enzyme in the production of prostanoids and since inflammatory pathways enhance the expression of an inducible COX (COX-2), both COX-2 and iNOS may be co-expressed in response to an inflammatory stimulus. Although regulation of the COX-2 pathway by NO has been demonstrated in animal models, its potential importance in human airway epithelium has not been investigated.

2 The effect of endogenous and exogenous NO on the COX-2 pathway was investigated in the A549 human airway epithelial cell culture model. Activity of the COX-2 pathway was assessed by PGE_2 EIA, and iNOS pathway activity by nitrite assay. A combination cytokine stimulus of interferon gamma (IFNγ) 100 u ml^{-1}, interleukin-1β (IL-1β) 1 u ml^{-1} and lipopolysaccharide (LPS) 10 μg ml^{-1} induced nitrite formation which could be inhibited by the competitive NOS inhibitor N^G-nitro-L-arginine-methyl-ester (L-NAME). IL-1β alone (1–50 u ml^{-1}) induced PGE_2 formation without significant nitrite formation, a response which was inhibited by the COX-2 specific inhibitor nimesulide. Submaximal stimuli used for further experiments were IFNγ 100 u ml^{-1}, IL-1β 1 u ml^{-1} and LPS 10 μg ml^{-1} to induce both the iNOS and COX-2 pathways, and IL-1β 3 u ml^{-1} to induce COX-2 without iNOS activity.

3 Cells treated with IFNγ 100 u ml^{-1}, IL-1β 1 u ml^{-1} and LPS 10 μg ml^{-1} for 48 h either alone, or with the addition of L-NAME (0 to 10^{-2} M), demonstrated inhibition by L-NAME of PGE_2 (3.61 ± 0.55 to 0.51 ± 0.04 pg/10^4 cells; $P < 0.001$) and nitrite (34.33 ± 8.07 to 0 pmol/10^4 cells; $P < 0.001$) production. Restoration of the PGE_2 response (0.187 ± 0.053 to 15.46 ± 2.59 pg/10^4 cells; $P < 0.001$) was observed after treating cells with the same cytokine stimulus and L-NAME 10^{-6} M, but with the addition of the NOS substrate L-arginine (0 to 10^{-5} M).

4 Cells incubated with IL-1β 3 u ml^{-1} for 6 h, either alone or with addition of the NO donor S-nitroso-acetyl-penicillamine (SNAP) (0 to 10^{-4} M), demonstrated increased PGE_2 formation (1.23 ± 0.03 to 2.92 ± 0.19 pg/10^4 cells; $P < 0.05$). No increase in PGE_2 formation was seen when the experiment was repeated in the presence of the guanylate cyclase inhibitor methylene blue (50 μM). Cells treated with SNAP alone did not demonstrate an increased PGE_2 formation. Cells incubated with IL-1β 3 u ml^{-1} for 6 h in the presence of dibutyryl cyclic guanylate monophosphate (0 to 10^{-3} M) also demonstrated an increased PGE_2 response (2.56 ± 0.21 to 4.53 ± 0.64 pg/10^4 cells; $P < 0.05$).

5 These data demonstrate that in a human airway epithelial cell culture system, both exogenous and endogenous NO increase the activity of the COX-2 pathway in the setting of inflammatory cytokine stimulation, and that this effect is likely to be mediated by guanylate cyclase. This suggests a role for NO in the regulation of human airway inflammation.

Keywords: Nitric oxide; inducible nitric oxide synthase; prostaglandin E_2; inducible cyclo-oxygenase; airway epithelium; inflammation

Introduction

The airway epithelium is recognised as an important regulatory system in mammalian airway physiology (Knight *et al*., 1994), and is thought to play an important role in the regulation of airway inflammation through the release of cytokines, the expression of cell adhesion molecules and the catabolism of neuropeptides (Knight *et al*., 1994; Thompson *et al*., 1995). It is now clear that the endogenous gas nitric oxide (NO), and the products of the cyclo-oxygenase pathway such as prostaglandin E_2 (PGE_2) are derived from the airway epithelium (Ozaki *et al*., 1987; Barnes, 1996). However, the importance of these mediators in regulating human airway function is yet to be fully characterized.

Nitric oxide (NO) is an endogenous short-lived free radical gas mediator involved in the regulation of vascular smooth muscle tone, inflammation, cell mediated immunity and coagulation (Nussler & Billiar, 1993). NO exerts its effects on cell function through a variety of interactions, including binding to heme-containing moieties of enzymes such as guanylate cyclase (Moncada & Higgs, 1993). The activation of guanylate cyclase and the resultant formation of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) is responsible for the activity of NO as a relaxant of vascular smooth muscle (Palmer *et al*., 1987). NO is synthesized by a family of nitric oxide synthases (NOS) and three distinct isoforms of this enzyme have been identified and cloned in man (Nathan & Xie, 1994). The activity of the inducible, calcium-independent form (iNOS) is predominantly regulated at a transcriptional level in response to stimuli such as pro-inflammatory cytokines (Nathan & Xie, 1994).

NO may be an important mediator in the context of airway inflammation (Gaston *et al*., 1994). Exhaled nitric oxide con-

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centrations in man are significantly increased in diseases such as asthma and bronchiectasis (Barnes & Kharitonov, 1996), and work in our laboratory (Watkins *et al*., 1997) and by others (Hamid *et al*., 1993; Kobzik *et al*., 1993; Guo *et al*., 1995) has localized iNOS mRNA and/or protein to the airway epithelium. In lower mammals, epithelial-derived NO appears to exert significant modulation of bronchoconstrictor responses both *in vivo* and *in vitro* (Nijkamp *et al*., 1993). Although inhaled NO has modest bronchodilator properties in man (Hogman *et al*., 1993), a role for epithelial derived NO in the regulation of human airway smooth muscle contractility has not been established. Furthermore, although many of the biological effects of NO on the inflammatory and immune systems are of direct relevance to airway disease (Gaston *et al*., 1994), the precise role of NO in human airway inflammation remains unknown.

Prostaglandins are lipid mediators derived from membrane phospholipids which regulate smooth muscle contractility, inflammation and platelet function (Sigal, 1991). Cyclo-oxygenase (COX), the first committed step in the prostanoid pathway, converts arachidonic acid to PGH_2 which is then converted to a variety of different prostanoids by the activity of tissue specific prostaglandin synthases (DeWitt & Smith, 1995). Two isoforms of COX have been identified (DeWitt & Smith, 1995). The constitutive form (COX-1) is associated with cellular homeostasis, whereas the inducible form (COX-2) is upregulated in response to inflammatory stimuli such as cytokines and lipopolysaccharide (LPS), in an analogous fashion to iNOS (DeWitt & Smith, 1995). Indeed, studies in animal (Vane *et al*., 1994) and cellular (Swierkosz *et al*., 1995) models of inflammation have demonstrated co-induction of the iNOS and COX-2 systems.

In mammalian airways, the predominant prostanoid product of the epithelial layer is PGE_2 (Churchill *et al*., 1989), which has important inhibitory effects on inflammatory cell activation and airway smooth muscle contractility (Pavord & Tattersfield, 1995). In human airways *in vitro*, we have shown that epithelial-derived PGE_2 modulates bronchoconstrictor responses, suggesting that PGE_2, rather than NO, may be the dominant epithelial modulator of human airway smooth muscle tone (Knight *et al*., 1992; 1994; 1995). Although both COX-1 and COX-2 have been identified in lung homogenates (O'Neill & Ford-Hutchison, 1994), definitive localization of both isoforms of COX in the human airway has not been performed. However, human cultured airway epithelial cells express both COX-1 and COX-2 isoforms (Mitchell *et al*., 1994), and synthesize PGE_2 (Ozaki *et al*., 1987).

Conflicting studies in both animal models and cell culture systems have demonstrated evidence for interactions between the NOS and COX pathways. In the setting of inflammatory induction of iNOS and COX-2, the majority of studies suggest that NO enhances COX-2 expression and activity (Salvemini *et al*., 1993; 1995; Sautebin & Di Rosa, 1994), and that this effect may be cyclic GMP-dependent (Tetsuka *et al*., 1996). In contrast, studies in LPS-activated rat macrophages have suggested that NO may inhibit both COX-2 induction and activity (Swierkosz *et al*., 1995). The interaction between the NOS and COX pathways has not been studied in human cell systems, or in the context of airway epithelial cell physiology. The aim of this study was to examine the effects of both endogenous and exogenous NO on the activity of the COX-2 pathway in a human airway epithelial cell line (A549), which has previously been shown to express both iNOS (Asano *et al*., 1994; Robbins *et al*., 1994) and COX-2 (Mitchell *et al*., 1994) activity.

Methods

Cell culture

A549 cells, a human airway epithelial carcinoma cell line with type II alveolar epithelial cell differentiation (Leiber, 1976), was obtained from American Type Culture Collection (Rockville, U.S.A.) and grown in a humidified 37°C environment in Ham's F12 nutrient mixture supplemented with 10% v/v FCS and gentamicin 50 μg ml^{-1}. When confluent, cells were dissaggregated in trypsin-versene solution, washed in F12/10% FCS, centrifuged at 100 *g* for 5 min, resuspended and subcultured according to standard protocols.

For cell pharmacology experiments, cells were seeded into 24 well tissue culture plates at a density of 5×10^4 cells/well, and grown to confluence (usually 48 h). Cells were then washed twice in 1X phosphate buffered saline (PBS) before incubation in 300 μl per well F12/1% FCS for the period of the experiment. Six wells were used for each experimental condition. At the conclusion of each experiment, supernatants were collected, centrifuged at 12,000 *g* for 5 min at 4°C, and stored at −80°C. Cells from each well were then treated with trypsin as described above, resuspended in 1X PBS and counted by trypan blue exclusion.

PGE_2 enzyme immunoassay

A competitive PGE_2 enzyme immunoassay (EIA) system was used according to the manufacturer's instructions and published methods (Pradelles *et al*., 1985). Unextracted cell free supernatants were assayed and blanks and standard curves were performed with F12/1% FCS as a diluent. Absorbance at 420 nm was read on a Spectramax 250 plate reader (Molecular Devices, Sydney, Australia). The manufacturer's specifications for this assay include an intra-assay coefficient of variation of 10%, cross-reactivity with PGD_2 and $PGF_{2\alpha}$ of less than 0.01%, and linearity over a range of 10–1000 pg ml^{-1}. Cell PGE_2 production was expressed as pg PGE_2 per 10^4 viable cells.

Nitrite assay

Nitrite, a stable breakdown product of NO in physiological systems, was assayed by use of the Griess reaction (Green *et al*., 1982). Cell culture supernatants (50 μl) were added to 50 μl of Griess reagent (sulphanilamide 1%, 0.1% naphthylethylenediamine 0.1%, phosphoric acid 2.5%) in duplicate on 96 well plates. After incubation at room temperature for 10 min, absorbance at 550 nm was read by a Spectramax 250 plate reader. Doubling dilutions of a 50 μM sodium nitrite solution in F12/1% FCS medium were used to generate a standard curve. Cell nitrite biosynthesis is expressed as pmol $NO_2^-/10^4$ viable cells.

Materials and reagents

Cell culture media and foetal calf serum (FCS) were purchased from GIBCO (Sydney, Australia), trypsin-versene solution from CSL (Perth, Australia), cell culture plasticware and disposables from Falcon (Perth, Australia), The PGE_2 monoclonal EIA reagents and COX inhibitors were supplied by Cayman Chemical (Ann Arbor, U.S.A.), and the recombinant human cytokines were purchased from Boehringer-Mannheim (Sydney, Australia). All remaining reagents and biochemicals were supplied by Sigma-Aldrich (Sydney, Australia).

Statistical analysis

Assay data were expressed as mean and s.e.mean. Further analysis was then performed by one way ANOVA and the Tukey-Kramer Multiple Comparisons test. A P value < 0.05 was considered significant.

Results

Characterization of iNOS induction by inflammatory cytokines

Cells were stimulated with a combination of interferon γ (IFNγ) 100 u ml^{-1}, tumour necrosis factor α (TNFα) 500 u

ml^{-1}, interleukin-1β (IL-1β) 10 u ml^{-1} and LPS 10 μg ml^{-1} for 12, 24 and 48 h. Nitrite concentrations in cytokine stimulated cells demonstrated a marked increase at 48 h (Figure 1a). In order to determine a submaximal stimulus for nitrite formation, cells were incubated with a variety of cytokine stimuli for 48 h (Figure 1b). A combination of IFNγ 100 u ml^{-1}, IL-1β 1 u ml^{-1} and LPS 10 μg ml^{-1} resulted in approximately 50 pmol NO_2^-/10^4 cells, which was 50% of the maximal cytokine response. With this submaximal stimulus, cytokine stimulation experiments were repeated in the presence of the competitive NOS inhibitor N^G-nitro-L-arginine-methyl-ester (L-NAME) at increasing concentrations (Figure 1c). Maximal inhibition of nitrite formation was observed at a concentration of 1 mM.

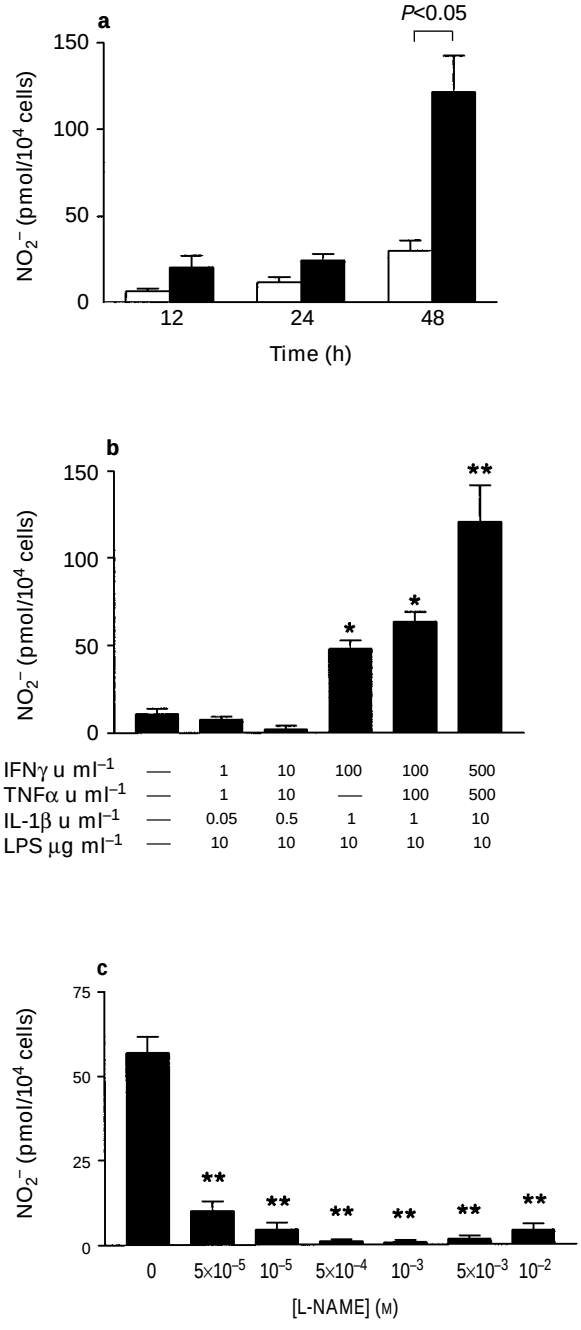

Figure 1 (a) Effect of cytokine stimulation on nitrite formation by A549 cells. Cells were treated with a combination of IFNγ 500 u ml^{-1}, TNFα 500 u ml^{-1}, IL-1β 5 u ml^{-1} and LPS 10 μg ml^{-1} (solid columns) or with media alone (open columns). (b) Effect of differing combinations of cytokines and LPS (shown below) on nitrite formation by A549 cells cultured for 48 h. $*P < 0.05$ $**P < 0.001$ compared to unstimulated cells. (c) Effect of L-NAME on nitrite formation by A549 cells cultured for 48 h. Cells were treated with IFNγ 100 u ml^{-1}, IL-1β 1 u ml^{-1} and LPS 10 μg ml^{-1} with the addition of increasing concentrations of L-NAME. $**P < 0.001$ compared to cells not treated with L-NAME.

Characterization of COX-2 induction by inflammatory cytokines

Cells were treated with increasing concentrations of IL-1β for 6 h (Figure 2a). Maximal PGE_2 production was observed in response to IL-1β at a concentration of 5 u ml^{-1}. The same supernatants were assayed for nitrite, and trace amounts were detected in only those cultures treated with IL-1β at concentrations above 50 u ml^{-1} (data not shown). In order to confirm that this IL-1β stimulus resulted in the induction of COX-2, the experiment was repeated by treating cells with IL-1β 3 u ml^{-1} in combination with increasing concentrations of nimesulide, a competitive COX-2 specific inhibitor (Vigdahl & Tukey, 1977). This demonstrated decreased PGE_2 supernatant concentrations with increasing concentrations of nimesulide (Figure 2b).

Co-induction of iNOS and COX-2 pathways

Cells were treated with a combination of IFNγ 100 u ml^{-1}, IL-1β 1 u ml^{-1} and LPS 10 μg ml^{-1} for 48 h and the supernatants assayed for both NO_2^- and PGE_2; both increased compared to control cells (Figure 3).

Effect of the iNOS inhibitor L-NAME on the co-activation of iNOS and COX-2 pathways

In order to examine the effects of endogenous NO on COX-2 activation by inflammatory cytokines, cells were treated with IFNγ 100 u ml^{-1}, IL-1β 1 u ml^{-1} and LPS 10 μg ml^{-1} for

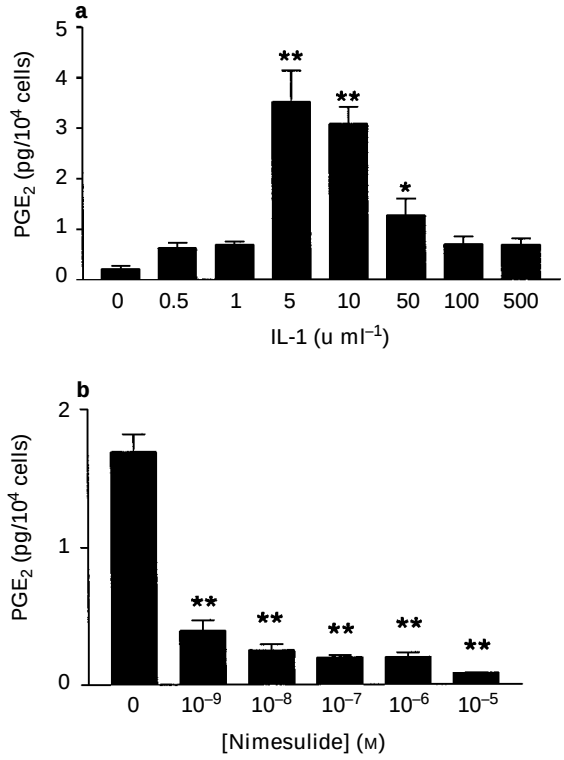

Figure 2 (a) Effect of IL-1β on PGE_2 formation by A549 cells cultured for 6 hours. $*P<0.05$ $**$ $P<0.001$ compared to unstimulated cells. (b) Effect of the COX-2 specific inhibitor nimesulide on IL-1β-induced PGE_2 formation in A549 cells cultured for 6 hours. Cells were treated with IL-1β 3 u ml^{-1} in combination with increasing concentrations of nimesulide. $**P<0.001$ compared to cells not treated with nimesulide.

48 h in the presence of increasing concentrations of L-NAME. Marked reductions in both PGE_2 and NO_2^- formation were seen (Figure 4). This experiment was repeated in the presence of L-NAME 1 mM and increasing concentrations of L-arginine. Restoration of the PGE_2 response to IFNγ/IL-1β/LPS was seen with L-arginine at a concentration above 10^{-6} M (Figure 5).

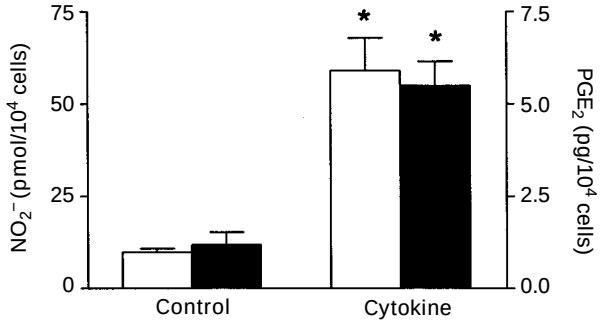

Figure 3 Effect of cytokine/LPS stimulation on both nitrite (solid columns) and PGE_2 (open columns) formation in A549 cells cultured for 48 h. Cells were treated with a combination of IFNγ 100 u ml^{-1}, IL-1β 1 u ml^{-1} and LPS 10 μg ml^{-1}. Control cells were treated with media only. $^*P < 0.05$ compared with control.

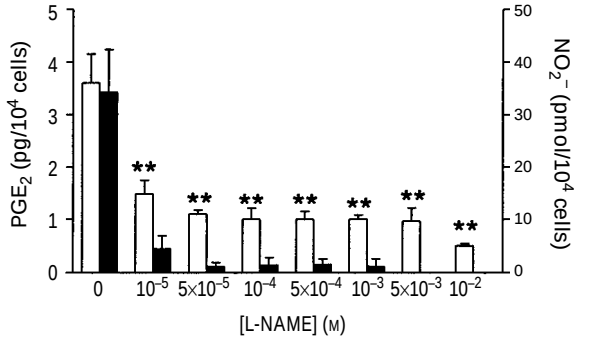

Figure 4 Effect of L-NAME on cytokine induced nitrite (solid columns) and PGE_2 (open columns) formation in A549 cells cultured for 48 h. Cells were treated with a combination of IFNγ 100 u ml^{-1}, IL-1β 1 u ml^{-1} and LPS 10 μg ml^{-1} in combination with increasing concentrations of L-NAME. $^{**}P<0.001$ compared with cells not treated with L-NAME.

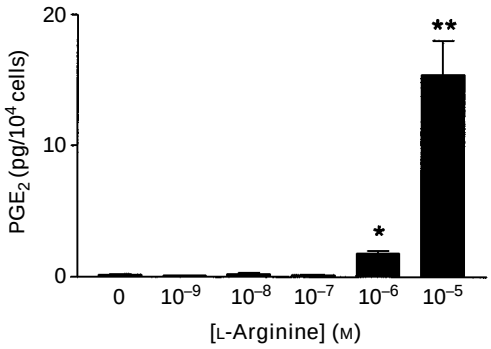

Figure 5 Effect of L-arginine on cytokine-induced PGE_2 formation by A549 cells cultured for 48 h in the presence of L-NAME. Cells were treated with a combination of IFNγ 100 u ml^{-1}, IL-1β 1 u ml^{-1}, LPS 10 μg ml^{-1} and L-NAME 1 mM in the presence of increasing concentrations of L-arginine. $^*P<0.05$, $^{**}P<0.001$ compared to cells not treated with L-arginine.

Effect of exogenous NO on the activation of COX-2 by IL-1β

In order to examine the effect of exogenous NO on cytokine-induced COX-2 expression and activity, a stimulus which induced PGE_2 formation without a detectable increase in supernatant nitrite concentration (IL-1β 3 u ml^{-1} for 6 h) was used. Cells were treated with IL-1β in the presence of increasing concentrations of S-nitroso-acetyl-penicillamine (SNAP), an NO donor. Significant increases in the PGE_2 response were seen with increasing concentrations of SNAP (Figure 6). The *in vitro* activity of SNAP was confirmed by a corresponding increase in supernatant NO_2^- concentration. The experiment was repeated without IL-1β, and no significant increase in PGE_2 concentration was observed (data not shown).

To determine whether the effects of exogenous NO are mediated by a cyclic GMP-dependent mechanism, cells were treated with IL-1β 3 u ml^{-1} in the presence of increasing concentrations of dibutyryl cyclic GMP, a stable cyclic GMP analogue (Rogers *et al*., 1988). A significant increase in PGE_2 production was seen in response to increasing concentrations of cyclic GMP in IL-1β treated cells, but not in unstimulated cells (Figure 7). The effect of the guanylate cyclase inhibitor methylene blue (Tetsuka *et al*., 1996) on cytokine-induced PGE_2 production in the presence of SNAP was also examined. Cells treated with IL-1β 3 u ml^{-1} in the presence of methylene blue 50 μM failed to show an increase in PGE_2 production in response to increasing concentrations of SNAP (Figure 8).

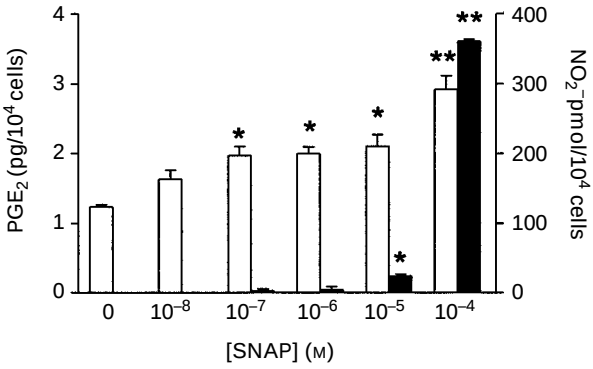

Figure 6 Effect of SNAP on IL-1β-induced nitrite (solid columns) and PGE_2 (open columns) formation by A549 cells cultured for 6 h. Cells were treated with IL-1β 3 u ml^{-1} in combination with increasing concentrations of SNAP. $^*P<0.05$, $^{**}P<0.001$ compared with cells not treated with SNAP.

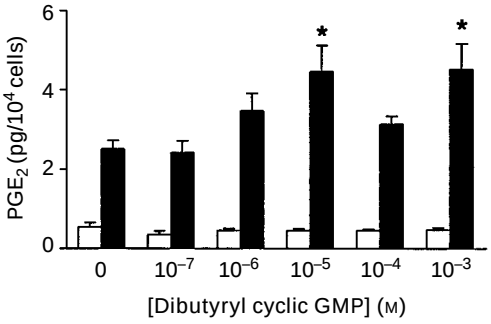

Figure 7 Effect of dibutyryl cyclic GMP (Bt_2cGMP) on PGE_2 formation by A549 cells cultured for 6 h. Cells were treated with IL-1β 3 u ml^{-1} (solid columns) or media only (open columns) in combination with increasing concentrations of dibutyryl cyclic GMP. $^*P<0.05$ compared to cells not treated with dibutyryl cyclic GMP.

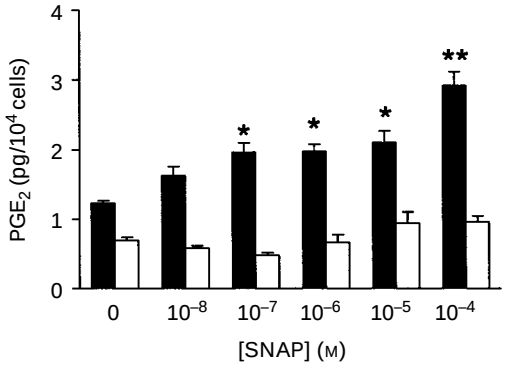

Figure 8 Effect of methylene blue on SNAP-mediated increases in IL-1β-induced PGE_2 formation by A549 cells cultured for 6 h. Cells were treated with IL-1β 3 u ml^{-1} alone (solid columns) or with methylene blue 50 μM (open columns). $^*P<0.05$, $^{**}P<0.001$ compared with cells not treated with SNAP.

Discussion

Although interactions between the prostanoid and NO pathways have been investigated in a limited number of animal models, this potentially important mechanism has not been characterized in human cell systems, or in respiratory epithelium. The purpose of our study was to investigate the hypothesis that NO is an important molecule in the regulation of the COX-2 pathway in respiratory epithelium with the A549 human cell culture model, which has previously been shown to express iNOS (Asano *et al*., 1994; Robbins *et al*., 1994) and COX-2 (Mitchell *et al*., 1994) activity in response to stimulation with inflammatory cytokines.

In our study, interactions between the iNOS and COX-2 pathways were examined by use of two approaches. Firstly, inhibition of endogenous NO production by the competitive NOS inhibitor L-NAME markedly reduced the PGE_2 response to an inflammatory cytokine stimulus which induced both iNOS and COX-2 activity, an effect which could be overcome by the addition of the NOS substrate L-arginine. To confirm the findings in the co-induction experiments, the effects of exogenous NO were then assessed by use of an NO donor (SNAP) in combination an IL-1β as stimulus which induced PGE_2, but not nitrite formation. These experiments demonstrated upregulation of IL-1β-induced PGE_2 formation by both NO and dibutyryl cyclic GMP, and that the NO effect was inhibited by methylene blue. These data suggest that NO upregulates the COX-2 pathway and that this effect is cyclic GMP-mediated.

In developing our experimental model, we confirmed data from a limited number of studies demonstrating that a combination cytokine stimulus induces iNOS (Asano *et al*., 1994; Robbins *et al*., 1994) or COX-2 (Mitchell *et al*., 1994) expression and activity in A549 cells.

Similarly, our data support the contention that in A549 cells, COX-2 induction occurs in response to stimulation with both IFNγ/IL-1β/LPS, and IL-1β alone in A549 cells (Mitchell *et al*., 1994). Although we did not specifically examine for evidence of iNOS or COX-2 gene expression, induction of these genes in response to cytokine stimulation is consistent both with our findings, and with published work in which the A549 cell culture model was used (Asano *et al*., 1994; Mitchell *et al*., 1994).

Co-induction of the iNOS and COX-2 pathways has been demonstrated in several animal models of inflammation both *in vitro* and *in vivo* (Corbett *et al*., 1993; Vane *et al*., 1994), and the relative activities of these inflammatory systems has been postulated as an important regulator of acute and chronic inflammation (Tetsuka *et al*., 1994). However, important species differences in the response have been demonstrated, with for example IL-1β activating both COX-2 and iNOS systems in rat, but not in human, cultured vascular smooth muscle cells (Lonchampt *et al*., 1994).

Studies in animal and cell culture models characterizing the interaction between the iNOS and COX-2 pathways by use of NOS inhibitors have demonstrated conflicting results. NOS inhibitors have been shown to inhibit cytokine-induced COX-2 expression and/or prostanoid formation in *in vivo* mammalian models (Corbett *et al*., 1993; Salvemini *et al*., 1994; 1995a; Sautebin & Di Rosa, 1994) and in cultured macrophage cell lines (Salvemini *et al*., 1993; Swierkosz *et al*., 1995). In contrast, a study of murine macrophages treated with a NOS inhibitor demonstrated an enhancement of LPS-induced COX-2 activity associated with an increase in the formation of $PGF_{2\alpha}$ (Swierkosz *et al*., 1995). Our data have confirmed that co-induction of iNOS and COX-2 activity occurs in response to a combination inflammatory cytokine stimulus, and have clearly demonstrated that in a human airway epithelial cell model, competitive inhibition of the iNOS system reduces cytokine-induced PGE_2 formation. This result suggests that endogenous NO formed as a consequence of the co-induction of both the iNOS and COX-2 systems may regulate COX-2 activity, and emphasizes the importance of both species and cell type in characterizing this interaction. An alternative explanation for these findings is that L-NAME exerts a direct effect on the COX-2 enzyme itself. However, this is unlikely based on our finding that the NOS substrate L-arginine reversed this effect of L-NAME on PGE_2 formation. Furthermore, work in cell free systems has clearly demonstrated that L-NAME does not inhibit COX-2 directly (Salvemini *et al*., 1995a).

An alternative strategy for the study of NO/COX interactions is to examine the effects of exogenous NO on cytokine-induced COX-2 activity. Studies in the rat forepaw model (Sautebin *et al*., 1995), and *in vitro* in primary rat mesangial cells (Tetsuka *et al*., 1996) have shown enhancement of cytokine-induced COX-2 activity by exogenous NO. Our experiments have demonstrated this effect in a human airway epithelial culture model, and in combination with our studies of L-NAME-induced inhibition of COX-2 activity, strongly suggest that in cultured A549 cells, NO has a significant regulatory effect on cytokine-induced prostanoid formation. Both SNAP and cyclic GMP alone, without a co-existent IL-1β stimulus, failed to result in an increase in PGE_2 formation, suggesting that NO in this situation acts as a modulator of the COX-2 pathway.

Activation of the COX-2 pathway by NO as demonstrated here, suggests a role for NO in amplifying regional inflammatory or anti-inflammatory responses. As the inhibitory prostaglandin PGE_2 is the major prostanoid product of the airway epithelium, the NO-COX interaction at the epithelial cell level may represent an important mechanism by which airway inflammatory responses are dampened, and has implications for the therapeutic use of inhaled NO in man.

The mechanism of interaction between NO and the COX pathways is not well characterized. Although direct interaction between the heme group of the COX-1 molecule and NO has been proposed (Salvemini *et al*., 1993; Tsai *et al*., 1994), the relevance of these findings to the effect of NO on the COX-2 system is unknown. In rat mesangial cell cultures, NO amplifies IL-1β-induced COX-2 mRNA expression and activity (Tetsuka *et al*., 1996), an effect which can be reproduced by cyclic GMP analogues. Our study did not directly address the level of the COX-2 pathway at which NO enhances PGE_2 production and we cannot exclude the possibility that NO amplifies the PGE_2 response by increasing the availability of arachidonic acid, or the activity of PGE_2 synthase. However, based on the effects seen with both dibutyryl cyclic GMP and methylene blue, our study strongly suggests that in A549 cells, a cyclic GMP-dependent mechanism is involved in the NO-mediated enhancement of cytokine-induced PGE_2 formation. Based on work in a rat mesangial cell model (Tetsuka *et al*., 1996), it seems likely that this occurs via an effect on the transcriptional activity of the COX-2 gene and suggests an

important role for NO in the regulation of gene expression in respiratory epithelium.

We have demonstrated a potentially important interaction between NO and the COX-2 pathway in human respiratory epithelium. In human airways, the potential regulation of epithelial PGE_2 production by NO may be of importance in modulating both airway contraction and inflammation. Such potential interactions require further investigations in physiological systems both *in vivo* and *in vitro*.

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