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Role of cyclo-oxygenase-2 induction in interleukin-1β induced attenuation of cultured human airway smooth muscle cell cyclic AMP generation in response to isoprenaline

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1 Airway smooth muscle (ASM) in human asthma shows reduced relaxation and cyclic AMP generation in response to β-adrenoceptor agonists. IL-β attenuates cyclic AMP generation but the underlying mechanism is unclear. We have reported that IL-1β induces cyclo-oxygenase-2 (COX-2) in human ASM cells and results in a marked increase in prostanoid generation with PGE_2 and PGI_2 as the major products.

2 We investigated the role of COX-2 induction and prostanoid release (measured as PGE_2) in IL-1β induced attenuation of cyclic AMP generation in response to the β-adrenoceptor agonist isoprenaline (ISO).

3 Pre-treatment of human ASM cells with IL-1β significantly attenuated cyclic AMP generation in response to high concentrations of ISO (1.0–10.0 μM) in a time- and concentration-dependent manner. The effect was accompanied by a high concentration of PGE_2 release. The non-selective COX inhibitor indomethacin (Ind), the selective COX-2 inhibitor NS-398, the protein synthesis inhibitors cycloheximide (CHX) and actinomycin D and the steroid dexamethasone (Dex) all abolished the PGE_2 release and prevented the attenuated cyclic AMP generation.

4 COX substrate arachidonic acid time- and concentration-dependently mimicked IL-1β induced attenuation and the effect was prevented by the non-selective COX inhibitors Ind and flurbiprofen, but not by NS-398, CHX and Dex.

5 In contrast to IL-1β, TNFα and IFNγ, which are ineffective in inducing COX-2 and releasing PGE_2 from human ASM cells, did not affect the cyclic AMP formation.

6 Our study demonstrates that COX-2 induction and the consequent release of prostanoids plays a crucial role in IL-1β induced attenuation of human ASM cell cyclic AMP response to ISO.

Keywords: β-adrenoceptor agonist; airway inflammation; airway smooth muscle; asthma; cyclic AMP; cyclo-oxygenase; interleukin-1β; isoprenaline; prostaglandin E_2; prostanoids

Introduction

Several pieces of evidence suggest that β-adrenergic relaxant mechanisms may be dysfunctional in asthmatic airways (Barnes, 1995). ASM relaxation to β-adrenoceptor agonists *in vitro* is impaired in airways taken from patients who died of asthma exacerbations (Bai, 1991; Goldie *et al*., 1986), in surgical lobectomy samples from patients with stable asthma (De Jongste *et al*., 1987) and in animal models of asthma (Barnes *et al*., 1980; Emala *et al*., 1993). The attenuated β-adrenergic responsiveness in asthmatic airways could not be attributed to a decrease in β-adrenergic receptor density or affinity (Sharma & Jeffery, 1990; Shore *et al*., 1997) or a specific defect in the receptor gene (Reihsaus *et al*., 1993). In one instance an increase in the receptor density and affinity was observed despite an impairment in functional relaxation (Spina *et al*., 1989). It has therefore been suggested that there is uncoupling of ASM β-receptors from adenylyl cyclase in asthma (Barnes, 1995).

Elevated levels of a number of pro-inflammatory cytokines, including interleukin-1β (IL-1β), tumour necrosis factor-α (TNFα) and interferon-γ (IFNγ), occur in bronchoalveolar lavage (BAL) fluid from asthmatic patients (Broide *et al*., 1992; Cembrzynska-Nowak *et al*., 1993). IL-1β and TNFα have been shown to play a major part in regulating smooth muscle contractility. IL-1β causes β-adrenergic hyporesponsiveness in various human and animal tissues and cells (Chung *et al*., 1990; Kelsen *et al*., 1995; Shore *et al*., 1997; Wills-Karp *et al*., 1993); treatment of rats *in vivo* and guinea-pig tracheas *in vitro* with TNFα also induces bronchial constrictor hyperresponsiveness (Kips *et al*., 1992) and reduces relaxation by β-adrenoceptor agonist isoprenaline (ISO) (Wills-Karp *et al*., 1993) respectively. Furthermore, IL-1β causes a decrease in response of human ASM cells to ISO (Shore *et al*., 1997) and TNFα inhibits ISO stimulated adenylyl cyclase activity in canine ASM cells (Emala *et al*., 1997). Taken together, these findings suggest that pro-inflammatory cytokines, particularly IL-1β, may be responsible in part for the reduced relaxant responses to β-adrenoceptor agonists in asthmatic patients and in animal models of asthma, but the precise mechanism(s) underlying this hyporesponsiveness has not been fully explored.

Cyclo-oxygenase (COX), the key enzyme converting arachidonic acid (AA) to prostanoids, exists in two isoforms. COX-1 is constitutively expressed whereas COX-2 is induced by inflammatory stimuli (Pang & Hoult, 1996; Pang & Knox, 1997; Vane, 1994). We and others have reported that IL-1β (Pang & Knox, 1997; Vigano *et al*., 1997) or a mixture of cytokines (Belvisi *et al*., 1997) induces COX-2 expression in cultured human ASM cells and the induction is accompanied by a marked increase in prostaglandin E_2 (PGE_2) and prostaglandin I_2 (PGI_2) production (Pang & Knox, 1997). Since PGE_2 and PGI_2 are both coupled to adenylyl cyclase and

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increase intracellular cyclic AMP, chronically elevated levels of PGE_2 and PGI_2 would be expected to cause heterologous desensitization of adenylyl cyclase. We postulated that IL-1β induced COX-2 expression and the consequent release of high concentration PGE_2 and PGI_2 are responsible for attenuating cyclic AMP generation in response to β-adrenoceptor agonists in IL-1β treated human ASM cells and impairing ASM relaxation in asthma.

The present study explores the potential link between IL-1β enhanced prostanoid release (measured as PGE_2) *via* COX-2 induction and the attenuation of human ASM cell cyclic AMP responses to ISO. The non-selective COX inhibitor indomethacin (Ind), selective COX-2 inhibitor NS-398 (N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulphonamide), protein synthesis inhibitors cycloheximide (CHX) and actinomycin D (Act) and the anti-inflammatory steroid dexamethasone (Dex) were used to assess the role of COX-2 activity and induction in the process. In addition, the ability of exogenously applied COX substrate arachidonic acid (AA) to mimic the attenuating effect of IL-1β was also investigated and the effect of IL-1β was compared to that of TNFα and IFNγ which are ineffective in COX-2 induction and prostanoid release in human ASM cells (Pang & Knox, 1997).

Methods

Cell culture

Human tracheas were obtained from two post-mortem individuals (one male aged 44 and one female aged 52, with no evidence of airway diseases) within 12 h of death. Primary cultures of human ASM cells were prepared from explants of ASM according to methods previously reported (Hall *et al*., 1992; Pang & Knox, 1997). Cells at passage three to four were used for all experiments. We have previously shown that the cells grown in this manner depict the immunohistochemical and light microscopic characteristics of typical ASM cells (Pang & Knox, 1997).

Experiment protocol

The cells were cultured to confluence in 24-well culture plates and growth-arrested in serum deprived medium for 24 h prior to experiments. Immediately before each experiment, fresh serum free medium containing the cytokines to be tested was added. In most experiments the cells were incubated with either 1.0 ng ml^{-1} IL-1β, or 10.0 ng ml^{-1} TNFα or 50.0 ng ml^{-1} IFNγ for 24 h. In the time course experiments the cells were incubated with IL-1β (1.0 ng ml^{-1}) for 1–24 h whereas in the concentration response experiments the cells were incubated for 24 h with 0.01–10.0 ng ml^{-1} IL-1β. At the indicated times, the culture media were either removed or harvested and stored at -20°C until the radioimmunoassay of PGE_2 content (Delamere *et al*., 1994). The anti-PGE_2 antiserum had negligible cross-reactivity in our hands (Pang & Knox, 1997). To test the inhibition of various reagents on the effect of IL-1β or the inhibition of the effect of isoprenaline by the selective β-adrenoceptor antagonist propranolol, all drugs were added 30 min prior to the addition of IL-1β or ISO respectively. Experiments with AA were conducted in the same way as IL-1β. The cytokines were dissolved in serum-free medium, all other agents were dissolved in dimethyl sulphoxide (DMSO, final concentration 1.0% v/v) except AA which was dissolved in ethanol (final concentration 1.0% v/v). In all the studies, a group of control cells were incubated with the vehicles used to dissolve the agents applied in the experimental cells for the same period of time.

Cyclic AMP assay

After the incubation with cytokines or AA and the removal of culture media, the cells were washed three times with phosphate buffered saline (PBS) and incubated in 0.5 ml fresh medium with 1.0 mM 3-isobutyl-1-methylxanthine (IBMX) to prevent cyclic AMP degradation. The cyclic AMP production reaction was initiated with the addition of ISO and was terminated 10 min later with 0.1 ml 30% (w/v) ice cold trichloroacetic acid which was then removed by amine/freon extraction (Khym, 1975), cyclic AMP content in the extract was determined by a protein binding assay (Gilman, 1970).

Cell viability

The toxicity of all the chemicals used in this study and their vehicles DMSO and ethanol to human ASM cells was determined by MTT assay (Pang & Knox, 1997). After 24 h incubation with the chemicals, 20 μl 5 mg ml^{-1} MTT [thiazolyl blue, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide] was added to the culture medium in 96 well plates and incubated for 1 h at 37°C. After removing the medium, 200 μl DMSO was added to solubilize the blue coloured tetrazolium and the plates were shaken for 5 min and the OD_{550} values were read in a microplate reader. Viability was set as 100% in control cells.

Materials

Recombinant human IL-1β, PGE_2, arachidonic acid, propranolol, indomethacin, flurbiprofen, dexamethasone, cycloheximide, actinomycin D, 3-isobutyl-1-methylxanthine, adenosine 3′:5′-cyclic monophosphate, protein kinase, 3′:5′-cyclic AMP dependent, MTT [thiazolyl blue, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide] and anti-PGE_2 serum were all purchased from Sigma (Poole, Dorset, U.K.); recombinant human TNFα and IFNγ from Genzyme (West Malling, Kent, U.K.); [5,6,8,11,12,14,15(n)-^{3}H] PGE_2 (specific activity 6.737 TBq mmol^{-1}) and [8-^{3}H] adenosine 3′:5′-cyclic phosphate (specific activity 1.04 TBq mmol^{-1}) from Amersham Life Science (Little Chalfont, Bucks, U.K.); NS-398 from Cayman Chemical (Ann Arbor, MI, U.S.A.); isoprenaline from Calbiochem-Novabiochem (La Jolla, CA, U.S.A.).

Statistical analysis

Data were expressed as means ± s.e.mean from n determinations (wells). Statistical analysis was performed by using the statistical software from SPSS Inc (SPSS Inc., 1996). Data were tested by one way analysis of variance followed by unpaired two-tailed t-test to determine the significant differences between means. The results were adjusted for multiple testing by using Bonferroni's correction. P values of less than 0.05 were accepted as statistically significant.

Results

Effect of IL-1β on cyclic AMP formation in response to ISO

The synthesis of cyclic AMP by human ASM cells in response to ISO and the effect of the selective β-adrenoceptor antagonist

propranolol (Prop) were studied first. Basal cyclic AMP levels were low in control cells (0.344 ± 0.05 pmol million^{-1} cells) and greatly increased after stimulation with ISO (10.0 μM, 10 min) which was concentration-dependently inhibited by Prop, indicating that the ISO response is working through the β-adrenoceptors (Figure 1). The capacity of human ASM cells to generate cyclic AMP in unstimulated conditions and after IL-1β pre-treatment was then examined. As shown in Figure 2, IL-1β (1.0 ng ml^{-1} for 24 h) significantly increased basal cyclic AMP levels (1.061 ± 0.336 pmol million^{-1} cells, $P<0.01$) and cyclic AMP formation in response to 0.1 μM ISO ($P<0.01$) but markedly reduced cyclic AMP formation in response to 1.0 and 10.0 μM ISO ($P<0.05$, $P<0.01$ respectively) (Figure 2). Human ASM cells pre-treated with IL-1β (1.0 ng ml^{-1}) showed a time-dependent decrease in cyclic AMP formation in response to ISO in the time course experiment (Figure 3A). The attenuation was significant after 2 h of incubation and peaked at 24 h. Treatment of the cells with various concentrations of IL-1β for 24 h also produced a concentration-dependent attenuation of cyclic AMP production to ISO (Figure 3B). The cyclic effect was significant from 0.1 ng ml^{-1} and maximal at 1.0 ng ml^{-1}.

Effect of various inhibitors on IL-1β induced PGE_2 release and attenuation in cyclic AMP formation

The effect of the non-selective COX inhibitor Ind, selective COX-2 inhibitor NS-398, protein synthesis inhibitors CHX and Act and the steroid Dex was assessed on IL-1β induced PGE_2 generation and attenuation in cyclic AMP production in response to ISO. Basal release of PGE_2 was low from untreated human ASM cells, IL-1β (1.0 ng ml^{-1}, 24 h) markedly increased PGE_2 synthesis and this was completely blocked by all inhibitors tested (all 1.0 μM, $P<0.001$, Figure 4A). The cyclic AMP production (Figure 4B) in response to ISO (10.0 μM) was significantly attenuated by IL-1β (1.0 ng ml^{-1}, 24 h, $P<0.01$) and this attenuation was prevented by Ind, CHX, Act, Dex ($P<0.01$) and NS-398 ($P<0.001$). In contrast, after the cells were incubated with the inhibitors themselves (without IL-1β), no significant changes in cyclic AMP production from the cells in response to ISO were observed (24.64 ± 4.20 pmol million^{-1} cells for Ind; 20.04 ± 2.54 pmol million^{-1} cells for NS-398; 21.13 ± 2.41 pmol million^{-1} cells for CHX; 16.91 ± 0.60 pmol million^{-1} cells for Act; 23.96 ± 3.93 pmol million^{-1} cells for Dex; and 19.29 ± 0.73 pmol million^{-1} cells for control cells). These results suggest that COX products after IL-1β pre-treatment, particularly those from the inducible COX-2, are involved in IL-1β induced attenuation of human ASM cell responses to ISO.

Effect of the COX substrate AA on PGE_2 release and cyclic AMP formation in response to ISO

In order to further clarify the role of COX products in IL-1β induced desensitization of the cell responses to ISO, we examined if exogenously applied AA could result in similar attenuation. AA was found to cause PGE_2 release in a time- (Figure 5A) and concentration-dependent (Figure 5B) manner, the highest concentration of PGE_2 was observed after 16 h of incubation with 10.0 μM AA. The PGE_2 release was accompanied by the attenuation of cyclic AMP production in response to ISO (10.0 μM), also in a time- (Figure 6A) and concentration-dependent (Figure 6B) manner. The pattern was similar to that of IL-1β (Figure 3A and B). The non-selective COX inhibitors Ind and flurbiprofen (Flu) strongly and concentration-dependently inhibited the PGE_2 release from AA (Figure 7A) whereas other inhibitors NS-398, CHX and Dex had no significant effect (Figure 7B). Responses to ISO were strongly reduced by AA pre-treatment (10.0 μM, 24 h) and this attenuation was again prevented by Ind and Flu in a concentration-dependent manner (Figure 8A). However, NS-398, CHX and Dex, even at higher concentrations for the latter two

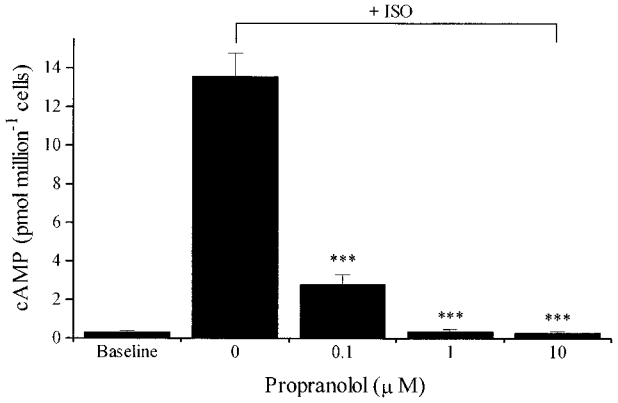

Figure 1 Effect of propranolol on cyclic AMP generation in response to isoprenaline (ISO). Confluent human ASM cells in 24-well plates were growth arrested for 24 h in serum-free medium. Fresh medium was changed before the experiments and the cells were incubated with increasing concentrations of propranolol in 0.5 ml medium for 30 min before further incubation with or without (baseline) 1.0 mM IBMX and 10.0 μM ISO for 10 min. The generated cyclic AMP was measured by a protein binding assay. Each point represents the mean $\pm$ s.e.mean of eight determinations from two independent experiments with cells from two individuals. Significant differences in cyclic AMP accumulation by propranolol-treated cells to vehicle-treated cells (0 μM propranolol) were calculated by ANOVA and an unpaired two-tailed t-test and adjusted by Bonferroni's correction for multiple comparisons (***$P<0.001$).

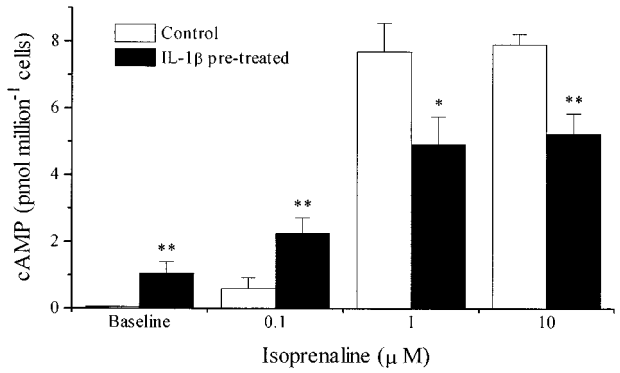

Figure 2 Concentration response of isoprenaline (ISO) on cyclic AMP generation from control cells and cells pre-treated with IL-1β. Confluent human ASM cells in 24-well plates were growth arrested for 24 h in serum-free medium. Fresh medium was changed before the experiments and the cells were incubated with or without IL-1β 1.0 ng ml^{-1} for 24 h. The medium was then removed and the cells were washed with PBS and incubated in 0.5 ml fresh medium with or without (baseline) 1.0 mM IBMX and various concentrations of ISO for 10 min. The generated cyclic AMP was measured by a protein binding assay. Each point represents the mean $\pm$ s.e.mean of eight determinations from two independent experiments with cells from two individuals. Significant differences in cyclic AMP accumulation by IL-1β-treated cells to vehicle-treated cells (control) were calculated by ANOVA and an unpaired two-tailed t-test and adjusted by Bonferroni's correction (*$P<0.05$, **$P<0.01$).

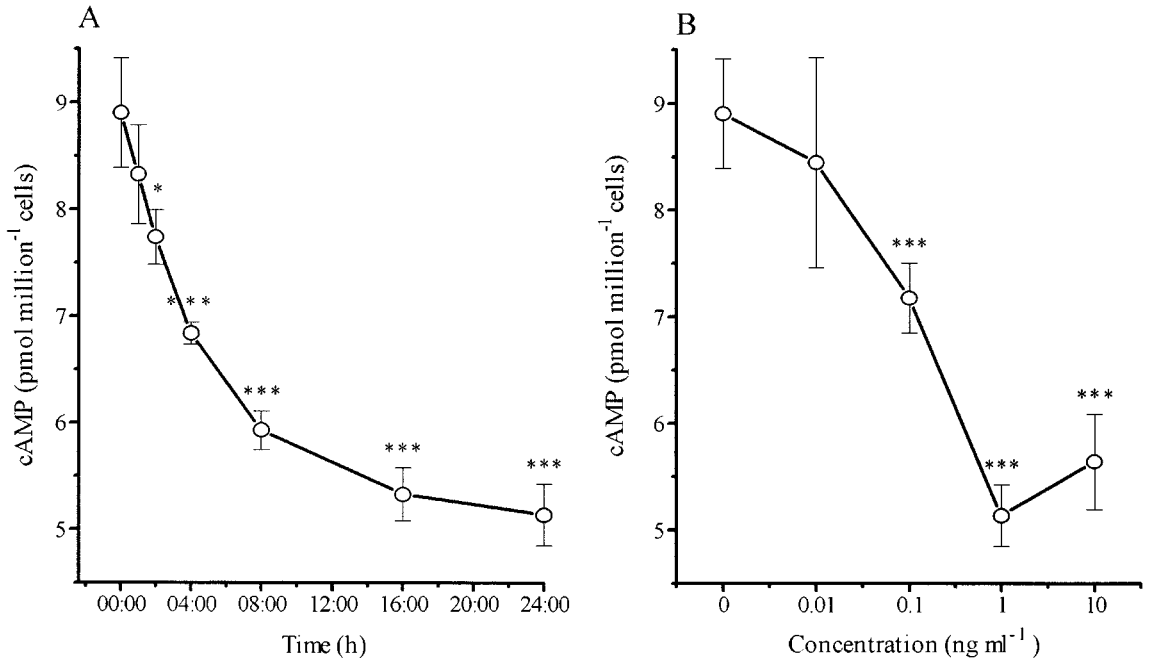

Figure 3 Time course (A) and concentration response (B) of IL-1β on cyclic AMP generation from human ASM cells in response to isoprenaline (ISO). Confluent human ASM cells in 24-well plates were growth arrested for 24 h in serum-free medium. Fresh medium was changed before the experiments and the cells were incubated with IL-1β (1.0 ng ml^{-1}) for the times indicated or with increasing concentrations of IL-1β for 24 h. The medium was then removed and the cells were washed with PBS and incubated in 0.5 ml fresh medium with 1.0 mM IBMX and 10.0 μM ISO for 10 min. The generated cyclic AMP was measured by a protein binding assay. Each point represents the mean$\pm$s.e.mean of eight determinations from two independent experiments with cells from two individuals. Significant differences in cyclic AMP accumulation by IL-1β-cyclic treated cells to vehicle-treated cells (control) were calculated by ANOVA and an unpaired two-tailed *t*-test and adjusted by Bonferroni's correction for multiple comparisons ($^{*}P<0.05$, $^{***}P<0.001$).

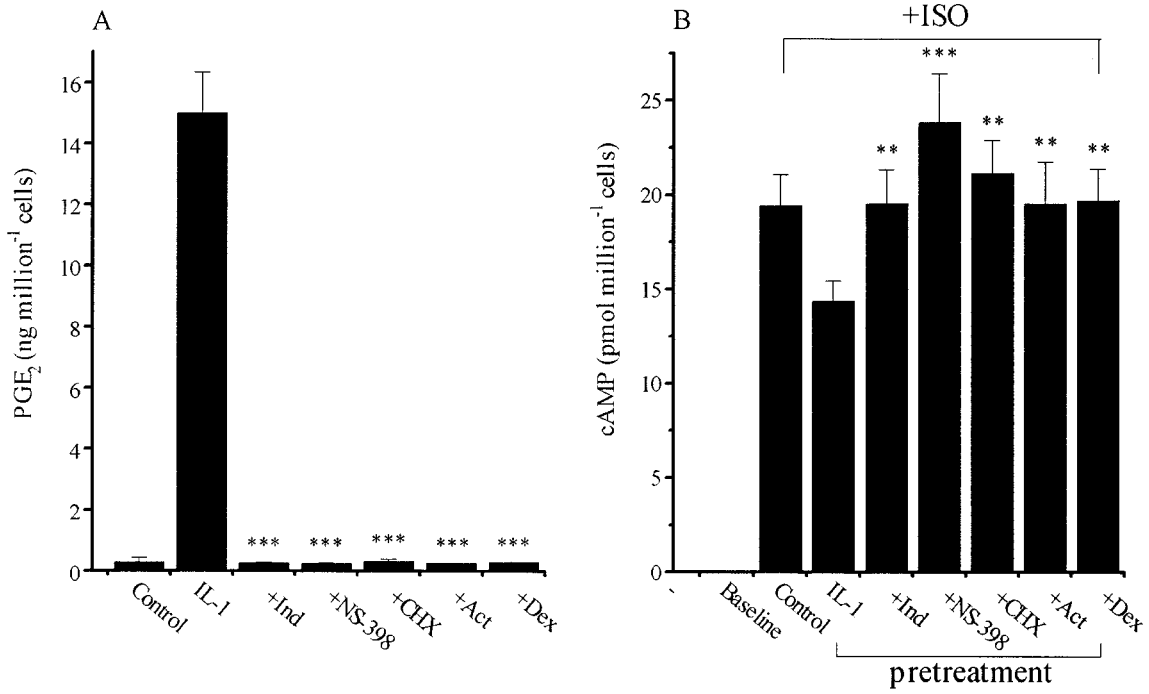

Figure 4 Effect of various inhibitors on IL-1β induced PGE_2 production (A) and cyclic AMP generation in response to isoprenaline (ISO) (B). Confluent human ASM cells in 24-well plates were growth arrested for 24 h in serum-free medium. Fresh medium was changed before the experiments and the cells were incubated with indomethacin (Ind), NS-398, cyclohexamide (CHX), actinomycin D (Act) or dexamethosone (Dex) (all 1.0 μM), respectively, for 30 min before the addition of IL-1β (1.0 ng ml^{-1}). After 24 h incubation the medium was removed for PGE_2 assay by RIA and the cells were washed with PBS and incubated in 0.5 ml fresh medium with or without (baseline) 1.0 mM IBMX and 10.0 μM ISO for 10 min. The generated cyclic AMP was measured by a protein binding assay. Each point represents the mean$\pm$s.e.mean of eight determinations from two independent experiments with cells from two individuals. Significant differences in PGE_2 and cyclic AMP accumulation by inhibitor-treated cells to IL-1β-treated cells were calculated by ANOVA and an unpaired two-tailed *t*-test and adjusted by Bonferroni's correction for multiple comparisons ($^{**}P<0.01$, $^{***}P<0.001$).

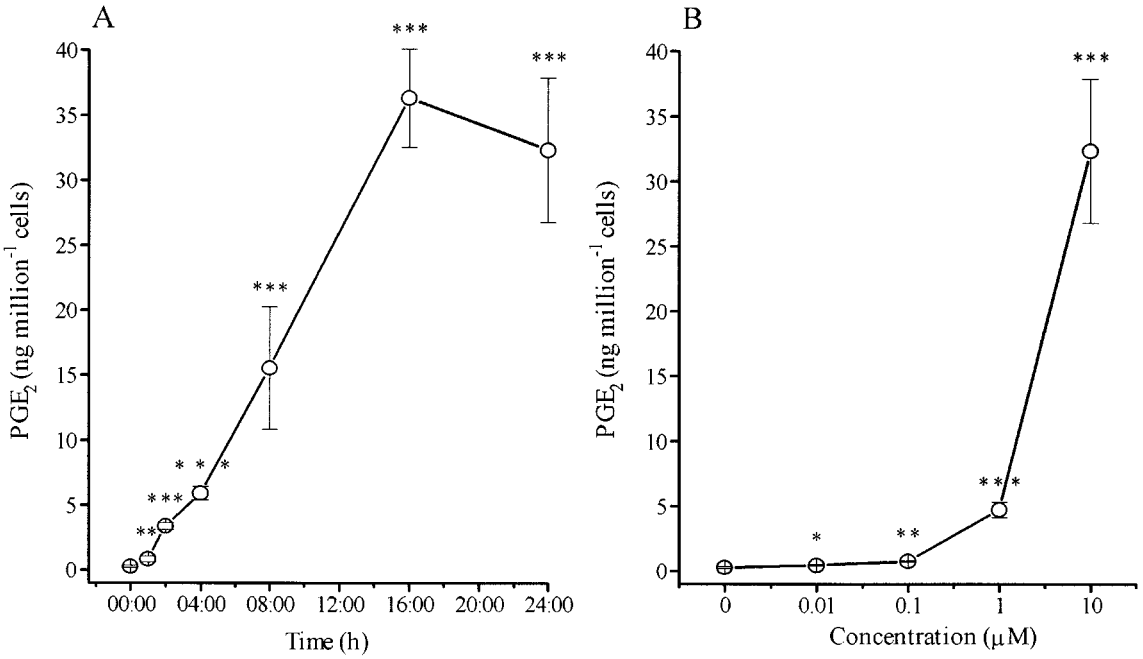

Figure 5 Time course (A) and concentration response (B) of arachidonic acid (AA) on PGE_2 production. Confluent human ASM cells in 24-well plates were growth arrested for 24 h in serum-free medium. Fresh medium was changed before the experiments and the cells were incubated with AA (10.0 μM) for the times indicated or with increasing concentrations of AA for 24 h. The medium was then removed for PGE_2 assay by RIA. Each point represents the mean ± s.e.mean of eight determinations from two independent experiments with cells from two individuals. Significant differences in PGE_2 release by AA-treated cells to vehicle-treated cells (control) were calculated by ANOVA and an unpaired two-tailed *t*-test and adjusted by Bonferroni's correction for multiple comparisons (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

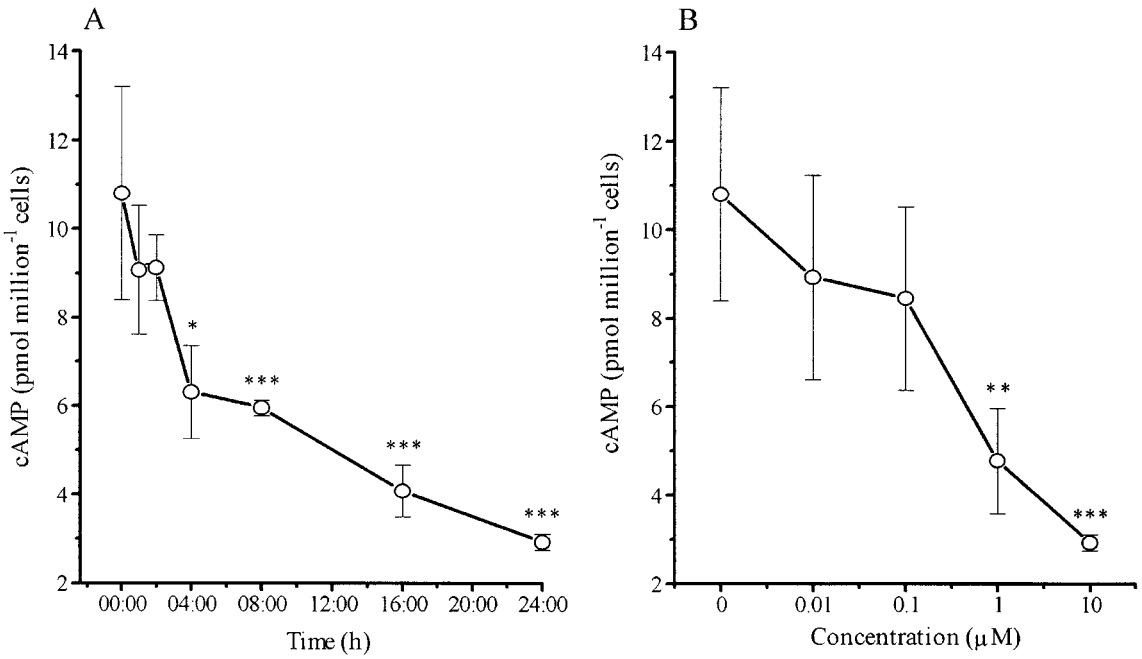

Figure 6 Time course (A) and concentration response (B) of arachidonic acid (AA) on cyclic AMP generation in response to isoprenaline (ISO). Confluent human ASM cells in 24-well plates were growth arrested for 24 h in serum-free medium. Fresh medium was changed before the experiments and the cells were incubated with AA (10.0 μM) for the times indicated or with increasing concentrations of AA for 24 h. The medium was then removed and the cells were washed with PBS and incubated in 0.5 ml fresh medium with 1.0 mM IBMX and 10.0 μM ISO for 10 min. The generated cyclic AMP was measured by a protein binding assay. Each point represents the mean ± s.e.mean of eight determinations from two independent experiments with cells from two individuals. Significant differences in cyclic AMP accumulation by AA-treated cells to vehicle-treated cells (control) were calculated by ANOVA and an unpaired two-tailed *t*-test and adjusted by Bonferroni's correction for multiple comparisons (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

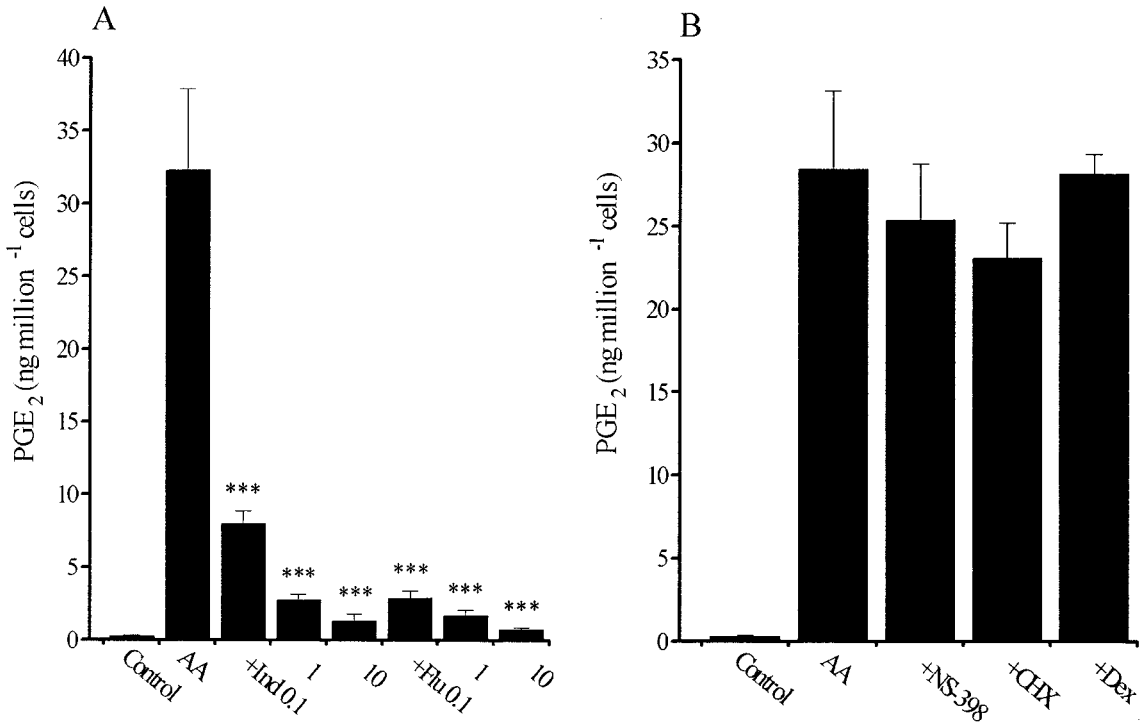

Figure 7 Effect of non-selective COX inhibitors (A) and other inhibitors (B) on AA induced PGE_2 production. Confluent human ASM cells in 24-well plates were growth arrested for 24 h in serum-free medium. Fresh medium was changed before the experiments and the cells were incubated with 0.1–10.0 μM indomethacin (Ind), 0.1–10.0 μM flurbiprofen (Flu), 1.0 μM NS-398, 10.0 μM cyclohexamide (CHX), or 10.0 μM dexamethasone (Dex), respectively, for 30 min before the addition of AA (10.0 μM). After 24 h incubation the medium was removed for PGE_2 assay by RIA. Each point represents the mean$\pm$s.e.mean of eight determinations from two independent experiments with cells from two individuals. Significant differences in PGE_2 production by inhibitor-treated cells to AA-treated cells were calculated by ANOVA and an unpaired two-tailed *t*-test and adjusted by Bonferroni's correction for multiple comparisons (***$P<0.001$).

(10.0 μM) than those used in the IL-1β experiment (1.0 μM, Figure 4B), were without effect (Figure 8B), indicating that COX products on their own mimic the attenuation effect of IL-1β and that COX-2 activity or induction is not involved in AA induced attenuation but is essential in IL-1β attenuated β-adrenergic responses.

Effect of TNFα and IFNγ on cyclic AMP formation in response to ISO

Since we have previously shown that TNFα and IFNγ have no effect on PGE_2 release and COX-2 induction in human ASM cells (Pang & Knox, 1997), we compared the effect of these cytokines with that of IL-1β on cyclic AMP production of the cells in response to ISO. As shown earlier (Figures 2, 3 and 4B), IL-1β caused a significant decrease in cyclic AMP production as compared with control cells. TNFα and IFNγ (24 h, 10.0 and 50.0 ng ml^{-1} respectively), however, had no effect (Figure 9), providing further evidence that COX-2 induction and the consequent release of COX products play a critical role in IL-1β induced desensitization of human ASM cell responses to ISO.

Cell viability

Cell viability for control cells was 100.00$\pm$9.68%. After 24 h treatment with AA 0.1, 1.0, 10 and 100 μM cell viability was 99.78$\pm$11.69%, 100.65$\pm$8.24%, 100.25$\pm$7.36% and 72.69$\pm$1.17% respectively. Due to the cytotoxic effect of 100 μM AA was used as the highest concentration in this study. Cell viability for drug vehicles 1% DMSO and 1% ethanol was 98.27$\pm$4.68% and 105.75$\pm$6.81% respectively. Cell viability after 24 h treatment with all other chemicals used in this study was consistently >95% compared with cells treated with the vehicles.

Discussion

Our results demonstrated that ISO stimulated large quantity cyclic AMP synthesis from human ASM cells *via* the activation of β-adrenoceptors; pre-treatment of human ASM cells with IL-1β resulted in a time- and concentration-dependent decrease in the responses of the cells to ISO and the attenuated response was prevented by reagents that blocked either the generation of COX products (by COX inhibitor Ind and COX-2 selective inhibitor NS-398) or the induction of COX-2 (by protein synthesis inhibitors CHX and Act and steroid Dex). Since we have recently reported details of the induction of COX-2 and prostanoid release in human ASM cells by IL-1β and the inhibition of this process by CHX, Act and Dex (Pang & Knox, 1997), the Western blot results were not shown here. The attenuating effect of IL-1β was mimicked by the COX substrate AA, which was then prevented by COX inhibitors Ind and Flu but was unaffected by NS-398, CHX and Dex. Furthermore, TNFα and IFNγ, which are ineffective on PGE_2 release and COX-2 induction in human ASM cells (Pang & Knox, 1997), had no effect on β-adrenergic responses in these cells. Our findings provide the first direct evidence that COX-2 induction and the consequent release of prostanoids play a

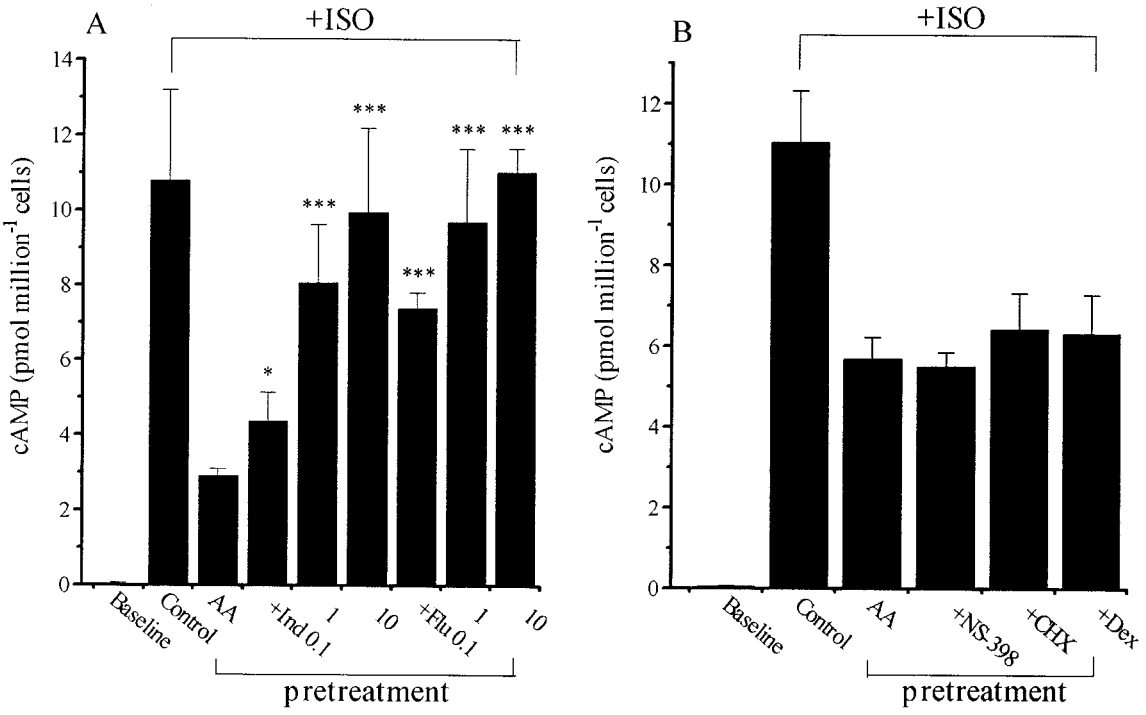

Figure 8 Effect of non-selective COX inhibitors (A) and other inhibitors (B) on AA induced cyclic AMP generation in response to isoprenaline (ISO). Confluent human ASM cells in 24-well plates were growth arrested for 24 h in serum-free medium. Fresh medium was changed before the experiments and the cells were incubated with 0.1–10.0 μM indomethacin (Ind), 0.1–10.0 μM flurbiprofen (Flu), 1.0 μM NS-398, 10.0 μM cycloheximide (CHX), or 10.0 μM dexamethasone (Dex), respectively, for 30 min before the addition of AA (10.0 μM). After 24 h incubation the medium was removed and the cells were washed with PBS and incubated in 0.5 ml fresh medium with or without (baseline) 1.0 mM IBMX and 10.0 μM ISO for 10 min. The generated cyclic AMP was measured by a protein binding assay. Each point represents the mean ± s.e.mean of eight determinations from two independent experiments with cells from two individuals. Significant differences in cyclic AMP accumulation by inhibitor-treated cells to AA-treated cells were calculated by ANOVA and an unpaired two-tailed *t*-test and adjusted by Bonferroni's correction for multiple comparisons (*$P<0.05$, ***$P<0.001$).

critical role in IL-1β induced hyporesponsiveness to β-adrenoceptor agonists.

Evidence has accumulated to support the hypothesis that cytokines, most notably IL-1β and to a lesser extent TNFα, contribute to the impaired airway relaxation in asthma. IL-1β causes β-adrenergic hyporesponsiveness in isolated guinea-pig (Wills-Karp *et al.*, 1993) and rabbit airways (Hakonarson *et al.*, 1996, 1997), human airway epithelial cells (Kelsen *et al.*, 1995) and human ASM cells (Shore *et al.*, 1997). Recently, Hakonarson and associates (Hakonarson *et al.*, 1997) demonstrated that the altered responsiveness of atopic/asthmatic sensitized rabbit ASM was largely attributed to autologously induced expression and autocrine action of IL-1β. The results in our present study with IL-1β are in agreement with the above findings, especially with those obtained from cultured human ASM cells. Several studies have evaluated the effect of TNFα on β-adrenergic-mediated responses in the airways. Treatment of rats *in vivo* with TNFα induced bronchial constrictor hyperresponsiveness (Kips *et al.*, 1992). TNFα also reduced relaxation by ISO in guinea-pig tracheas (Wills-Karp *et al.*, 1993) and rabbit trachea smooth muscle segments (Hakonarson *et al.*, 1996). The latest studies have further demonstrated that TNFα inhibits ISO-stimulated adenylyl cyclase activity in canine ASM cells (Emala *et al.*, 1997). However, unlike the above findings with TNFα, in the present study in human ASM cells we were unable to detect any substantial change in responses to ISO after treatment

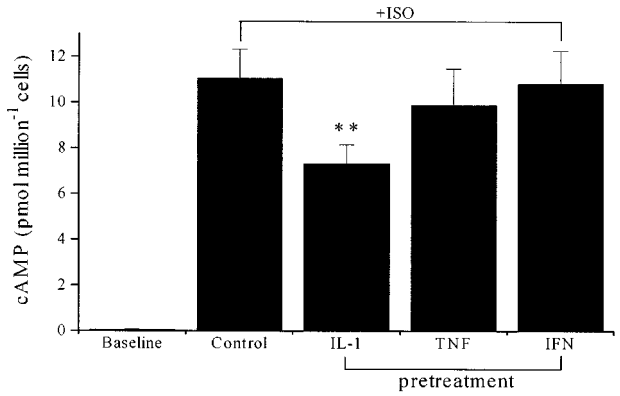

Figure 9 Effect of IL-1β, TNFα and IFNγ on cyclic AMP generation in response to isoprenaline (ISO). Confluent human ASM cells in 24-well plates were growth arrested for 24 h in serum-free medium. Fresh medium was changed before the experiments and the cells were incubated with IL-1β (1.0 ng ml^{-1}), TNFα (10.0 ng ml^{-1}) or IFNγ (50.0 ng ml^{-1}) for 24 h. The medium was then removed and the cells were washed with PBS and incubated in 0.5 ml fresh medium with or without (baseline) 1.0 mM IBMX and 10.0 μM ISO for 10 min. The generated cyclic AMP was measured by a protein binding assay. Each time point represents the mean ± s.e.mean of eight determinations from two independent experiments with cells from two individuals. Significant differences in cyclic AMP accumulation by cytoline-treated cells to vehicle-treated cells (control) were calculated by ANOVA and an unpaired two-tailed *t*-test and adjusted by Bonferroni's correction for multiple comparisons (**$P<0.01$).

with TNFα. The discrepancy could be explained by the difference in the tissue species and the culture conditions. In the study with canine ASM cells, significant changes could only be observed after 72 h but not 24 h incubation with TNFα (Emala *et al*., 1997), while in our study the cells were stimulated with TNFα for 24 h. Nevertheless, the negative results with TNFα and IFNγ provide complementary evidence to support our hypothesis that IL-1β induced impairment in human ASM cell responses to ISO is mainly mediated by COX-2 induction.

In airway smooth muscle, ISO binds to the β-adrenergic receptors that couple to the stimulatory G-protein G_s, the α subunit of which in turn activates the enzyme adenylyl cyclase to generate cyclic AMP (Barnes, 1995). Increased cyclic AMP activates protein kinase A and protein kinase C to cause relaxation of ASM (Barnes, 1995; Torphy, 1994). The observations that IL-1β did not affect the ability of direct adenylyl cyclase activator forskolin to cause cyclic AMP accumulation (Hakonarson *et al*., 1996; Shore *et al*., 1997) or smooth muscle relaxation (Hakonarson *et al*., 1996) suggest that the decreased β-adrenergic responsiveness by IL-1β is not due to any change in the activity or expression of adenylyl cyclase. The fact that a phosphodiesterase inhibitor IBMX was used together with ISO (Shore *et al*., 1997, this paper) also excludes the involvement of changes in phosphodiesterase activity in the effects of IL-1β within the constraint of our experimental design. It does not rule out an additional effect on phosphodiesterase. The effects in our experiments are likely to be mediated upstream of the adenylyl cyclase enzyme. The observations of others allow speculation on the interaction site. Further studies have shown that IL-1β had no effect on the expression of the stimulatory G-protein subunit $G_s\alpha$ (Shore *et al*., 1997), however, IL-1β attenuated relaxation of trachea smooth muscle to ISO was ablated by a muscarinic M_2-receptor antagonist and was associated with enhanced induction of the inhibitory G-protein subunits $G_i\alpha_2$ and $G_i\alpha_3$ (Hakonarson *et al*., 1996). This suggests that the cytokine induced impairment of airway responsiveness to β-adrenoceptor agonists may be in part attributable to enhanced M_2-receptor/G_i protein-coupled inhibition of adenylyl cyclase. Other mechanisms may also be involved however. Although our studies convincingly show that cyclic AMP accumulation is impaired by IL-1β, future studies are required to investigate the functional consequences of this in human ASM.

Prostaglandins (mainly PGE_2 and PGI_2) activate the prostaglandin EP2 and EP4 receptors, which are also coupled to G_s and adenylyl cyclase (Narumiya, 1994), to increase cyclic AMP production in human ASM cells (Hall *et al*., 1992). Thus PGs share a similar receptor-mediated signal transduction system with β-adrenoceptor agonists and the functional responses to PGE_2 receptor stimulation, like that to β-adrenoceptor stimulation, are attenuated by the activation of G_i protein (Lerner *et al*., 1992). It is therefore reasonable to speculate that elevated levels of PGs could cause heterologous attenuation of adenylyl cyclase. In fact, IL-1β caused a decreased response not only to ISO but also to PGE_2 (Shore *et al*., 1997; Hakonarson *et al*., 1996). Observations from our previous study and present study have demonstrated that IL-1β caused induction of COX-2 in human ASM cells and consequently resulted in marked increase in prostanoid generation with PGE_2 and PGI_2 as the major products (Pang & Knox, 1997); IL-1β attenuated the capacity of human ASM cells to form cyclic AMP in responses to ISO and this attenuation was prevented by reagents that blocked either the activity or the induction of COX-2 (this paper); exogenously applied AA, which in turn was converted to prostanoids by existing COX-1, also caused attenuated responses in a similar pattern as IL-1β, and exogenously applied PGE_2 concentration-dependently caused attenuated cyclic AMP responses to both ISO and PGE_2 (Pang *et al*., 1998). The findings are in agreement with the report that pre-treating human ASM cells with agents that induce cyclic AMP formation resulted in a marked decrease in the capacity of the cells to produce cyclic AMP following subsequent application of ISO (Hall *et al*., 1993). It is therefore likely that IL-1β induced decrease in responses of ASM to β-adrenoceptor agonists though the uncoupling of β-receptors from adenylyl cyclase activation may largely be mediated by the induction of COX-2.

In summary, we examined the role of COX-2 induction in IL-1β induced attenuation of human ASM cell cyclic AMP responses to the β-adrenoceptor agonist ISO. Our results demonstrated that: (a) pre-treatment of human ASM cells with IL-1β resulted in marked time- and concentration-dependent decrease in cyclic AMP accumulation following subsequent application of ISO; (b) agents that blocked either the activity or the induction of COX-2 not only abolished IL-1β induced PGE_2 generation but also prevented IL-1β induced attenuation of the cell cyclic AMP responses to ISO; (c) pre-treatment with COX substrate AA also caused impairment on cyclic AMP accumulation of the cells in a similar pattern as that of IL-1β and only agents that blocked the activity of the existing COX-1 prevented the attenuated response; (d) pre-treatment with TNFα and IFNγ, which do not cause COX-2 induction or prostanoid release in these cells, did not affect the cyclic AMP formation. Collectively, these findings indicate for the first time that COX-2 induction and the consequent release of prostanoids are fundamental in mediating IL-1β induced attenuation of human ASM cell cyclic AMP responses to ISO, thus providing fresh insights into the mechanisms of cytokine action in asthma pathogenesis.

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