British Journal of Pharmacology (1997) 120, 910–916

Induction of cyclo-oxygenase-2 by cytokines in human cultured airway smooth muscle cells: novel inflammatory role of this cell type

*,[1]Maria G. Belvisi, *Michael A. Saunders, *El-Bdaoui Haddad, †Stuart J. Hirst, ††Magdi H. Yacoub, *Peter J. Barnes & *,#Jane A. Mitchell

*Thoracic Medicine, Imperial College of Medicine at the National Heart & Lung Institute, Dovehouse Street, London SW3 6LY; †Department of Medicine, United Medical Schools of Guy's and St Thomas' Hospitals, St Thomas' Hospital, London SE1 7EH; ††Cardiothoracic Surgery, Imperial College School of Medicine at the National Heart & Lung Institute, Dovehouse Street, London SW3 6LY and #Department of Anaesthesia and Critical Care Medicine, The Royal Brompton Hospital, Sydney Street, London SW3 6NP

1 Cyclo-oxygenase (COX) is the enzyme that converts arachidonic acid to prostaglandin H_2 (PGH_2) which can then be further metabolized to prostanoids which modulate various airway functions. COX exists in at least two isoforms. COX-1 is expressed constitutively, whereas COX-2 is expressed in response to pro-inflammatory stimuli. Prostanoids are produced under physiological and pathophysiological conditions by many cell types in the lung. However, the regulation of the different COX isoforms in human airway smooth muscle (HASM) cells has not yet been determined.

2 COX-1 and COX-2 protein were measured by Western blot analysis with specific antibodies for COX-1 and COX-2. COX-2 mRNA levels were assessed by Northern blot analysis by use of a COX-2 cDNA probe. COX activity was determined by measuring conversion of either endogenous or exogenous arachidonic acid to three metabolites, PGE_2, thromboxane B_2 or 6-keto$PGF_{1\alpha}$ by radioimmunoassay.

3 Under control culture conditions HASM cells expressed COX-1, but not COX-2, protein. However, a mixture of cytokines (interleukin-1β (IL-1β), tumour necrosis factor α (TNFα) and interferon γ (IFNγ) each at 10 ng ml^{-1}) induced COX-2 mRNA expression, which was maximal at 12 h and inhibited by dexamethasone (1 μM; added 30 min before the cytokines). Furthermore, COX-2 protein was detected 24 h after the cytokine treatment and the expression of this protein was also inhibited by dexamethasone (1 μM) and cyclohexamide (10 μg ml^{-1}; added 30 min before the cytokines).

4 Untreated HASM cells released low or undetectable amounts of all COX metabolites measured over a 24 h period. Incubation of the cells with the cytokine mixture (IL-1β, TNFα, IFNγ each at 10 ng ml^{-1} for 24 h) caused the accumulation of PGE_2 and 6-keto-$PGF_{1\alpha}$.

5 In experiments where COX-2 metabolized endogenous stores of arachidonic acid, treatment of HASM cells with IL-1β in combination with TNFα caused a similar release of PGE_2 to that when the three cytokines were given in combination.

6 In other experiments designed to measure COX-2 activity directly, cells were treated with cytokines for 24 h before fresh culture medium was added containing exogenous arachidonic acid (30 μM for 15 min) after which PGE_2 was measured. IL-1β and TNFα increased COX-2 activity and an additional small increase was produced by the three cytokines in combination.

7 These findings suggest that the increased expression of COX-2 is intimately involved in the exaggerated release of prostanoids from HASM cells exposed to pro-inflammatory cytokines. These data indicate a role for airway smooth muscle cells, in addition to their contractile function, as inflammatory cells involved in the production of mediators which may contribute to the inflammatory response seen in diseases such as asthma.

Keywords: Prostaglandins; airway smooth muscle; asthma; cytokines; cyclo-oxygenase-2

Introduction

Cyclo-oxygenase (COX) is the enzyme which converts arachidonic acid to prostaglandin H_2 (Hamberg *et al*., 1974) which can be further metabolized to prostanoids, including prostaglandin E_2 (PGE_2), prostacyclin (PGI_2) and thromboxane A_2 (TXA_2). It is now known that COX can exist in at least two distinct isoforms (Xie *et al*., 1991; see Mitchell *et al*., 1995). COX-1 is generally thought to produce prostanoids which serve to maintain cellular homeostasis and is known to be expressed constitutively in many cell types including endothelial cells (Mitchell *et al*., 1995). COX-2 has been demonstrated to be expressed in response to many pro-inflammatory stimuli, including cytokines (Maier *et al*., 1990; Mitchell *et al*., 1994), in cells *in vitro* and at the site of inflammation *in vivo* (Vane *et al*., 1994). Furthermore, COX-2 is thought to be the isoform responsible for the production of pro-inflammatory prostanoids in various models of inflammation (Chan *et al*., 1995).

Prostanoids are produced both under physiological and pathophysiological conditions by all cell types present in the lung and are known to modulate various airway functions including airway and vascular tone, cell proliferation, plasma exudation, inflammatory cell recruitment and activity, cytokine release, mucus secretion and parasympathetic and sensory nerve function. However, the role of prostanoids in the lung is dependent on their local concentration, the profile of prostanoids released and the presence of specific cell surface receptors which are capable of activating different signal transduction pathways and therefore eliciting a wide range of effects.

[1] Author for correspondence.

Cytokine levels are increased in inflammatory airway diseases such as asthma (Barnes, 1994). Lipopolysaccharide (LPS) and/or certain cytokines, such as interleukin-1β (IL-1β) result in the increased expression of COX-2 in airway epithelial cells (Mitchell *et al*., 1994; Newton *et al*., 1996), airway macrophages (Lee *et al*., 1992; O'Sullivan *et al*., 1992; Mitchell *et al*., 1993; Hempel *et al*., 1994) and monocytes (Hempel *et al*., 1994). However, although prostanoids are known to be produced by airway smooth muscle cells (Delamere *et al*., 1994), the production of prostanoids under pro-inflammatory conditions and the regulation of the different isoforms of COX in these cells has not yet been determined. A preliminary account of this work was presented at a British Pharmacological Society meeting (Saunders *et al*., 1996).

Methods

Isolation of human airway smooth muscle cells

Cells were isolated and cultured as previously described (Hirst *et al*., 1992). Tracheal rings, obtained from either heart or heart and lung transplantation donors (2 female, 2 male, aged 24–37 years), were placed in sterile Hank's buffered salt solution (HBSS) (in mM: NaCl 136.8, KCl 5.4, $MgSO_4$ 0.8, Na_2HPO_4. $7H_20$ 0.4, $CaCl_2$. $2H_20$ 1.3, $NaHCO_3$ 4.2 and glucose 5.6) containing the antibiotics penicillin (100 u ml^{-1}), streptomycin (100 μg ml^{-1}) and the anti-fungal agent amphotericin B (2.5 μg ml^{-1}). With sterile instruments and under aseptic conditions the smooth muscle layer was dissected free of adherent connective tissue. A strip of smooth muscle (0.5–1 cm long) was dissected from between the cartilage plates and the epithelial layer removed by a rounded scalpel blade and incubated for 30 min at 37°C in 5% CO_2/air in HBSS containing 10 mg ml^{-1} bovine serum albumin (BSA) and the enzymes collagenase (type XI, 1 mg ml^{-1}) and elastase (type I, 3.3 u ml^{-1}). After further removal of remaining connective tissue, the smooth muscle was chopped finely and incubated for a further 150 min in the enzyme solution outlined above with elastase content increased to 15 u ml^{-1}. In order to separate the dispersed cells from the enzyme solution it was centrifuged (100 *g*, 5 min) at 4°C and then resuspended in Dulbecco's modified Eagle's medium (DMEM) containing heat inactivated foetal calf serum (10% v/v), sodium pyruvate (1 mM), L-glutamate (2 mM), non-essential amino acids (1x) and anti-microbial agents as previously described.

Primary culture of human airway smooth muscle (HASM) cells

The HASM cellular suspension was placed in a tissue culture flask (75 cm^2) with 6 ml of supplemented DMEM and incubated at 37°C in 5% CO_2/air. The culture medium was replaced after 4–5 days (12 ml) and subsequently every 3–4 days. After approximately 10–14 days the cells reach confluence at which time they were passaged by standard cell culture techniques into 2 × 75 cm^2 flasks which were split 1:2 at confluence. With this approach, cells could be maintained in culture over many passages (usually 4 to 12) (Hirst *et al*., 1992).

Cell counts were performed with a Neubauer haemocytometer; cell viability was assessed by trypan blue exclusion and was found to be greater than 97%. By use of immunofluorescence techniques, cultured HASM cells at confluence and in the absence of growth factors stained (<95%) for smooth muscle α-actin. In addition, when examined by light microscopy these cells displayed the characteristics of viable smooth muscle cells in culture (Twort & van Breemen, 1988). Cell respiration, an indicator of cell viability, was also assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan as previously described (Hirst *et al*., 1992; Mitchell *et al*., 1993). Cells were plated at a density of approximately 60,000 and 2000 cells/well onto 6 and 96 well culture plates, respectively. At confluence the cells were growth arrested, being placed in a serum free medium containing the supplements outlined above and apotransferin (5 μg ml^{-1}), insulin (1 μM), ascorbate (100 μM) and bovine serum albumin (0.1%). The cells were maintained in this medium for 72 h before the experiment was started. In experiments designed to investigate the effect of serum on cytokine (cytokine mixture: IL-1β, TNFα, IFNγ each at 10 ng ml^{-1})-induced PGE_2 release, 2.5% serum was found to enhance release optimally with no further increase observed at 10% (from 0.285 ± 0.098 ng ml^{-1} to 7.084 ± 0.749 ng ml^{-1} in cells treated with 2.5% serum; $n = 3$). All experiments, unless otherwise stated were performed with cells which were incubated with medium supplemented with 2.5% foetal calf serum.

Measurement of COX activity

COX metabolites were measured by radioimmunoassay (Salmon, 1978; Mitchell *et al*., 1994) for PGE_2, 6-keto $PGF_{1\alpha}$ and TXB_2. Antibodies to PGE_2 and TXB_2 had less than 5% cross-reactivity with all other prostaglandins measured. Antibody to 6-keto- $PGF_{1\alpha}$ had less than 5% cross reactivity to thromboxane B_2 and 17% cross reactivity to PGE_2. For experiments designed to measure the release of prostanoids from endogenous arachidonic acid experiments were performed with similar protocols to those previously described by Mitchell *et al*. (1994) for airway epithelial cells. Cells were grown in 96 well plates and treated with either lipopolysaccharide (LPS; 0.1–10 μg ml^{-1}), tumour necrosis factor-α (TNFα; 0.1–100 ng ml^{-1}), interleukin 1β (IL-1β; 0.1–100 ng ml^{-1}), interferon γ (IFNγ; 0.1–100 ng ml^{-1}), alone or in various combinations for 24 h and the medium removed for radioimmunoassay. In some experiments, dexamethasone (1 μM), cyclohexamide (10 μg ml^{-1}) or indomethacin (30 μg ml^{-1}) were added 30 min before the addition of the cytokine mixture (IL-1β, TNFα, IFNγ, all cytokines at 10 ng ml^{-1}) for 24 h and the predominant COX metabolite (PGE_2) measured. Indomethacin (30 μg ml^{-1}) was used at a concentration that is known to inhibit maximally both isoforms of COX (Mitchell *et al*., 1993). In separate experiments, designed to measure COX activity, cells were treated with different combinations of cytokines as detailed above for 24 h after which time fresh medium was added containing arachidonic acid (30 μM) for 15 min at 37°C and then removed for radioimmunoassay.

In another series of experiments, cells were exposed to the cytokine mixture (IL-1β, TNFα, IFNγ each at 10 ng ml^{-1}) for 0, 2, 4, 6, 12, 24 and 48 h and the medium removed for radioimmunoassay in order to assess the time-dependent release of prostanoids.

Western blot analysis

In other experiments HASM cells were grown in 6 well plates as detailed above. After exposure to the mixture of cytokines (IL-1β, TNFα, IFNγ 10 ng ml^{-1} for each; (±)-dexamethasone, 1 μM; indomethacin, 30 μg ml^{-1}; cyclohexamide, 10 μg ml^{-1}) the culture medium was removed, PGE_2 levels measured and the cells incubated with extraction buffer (Tris, 50 mM; EDTA,10 mM; Triton X-100, 1% v/v; phenylmethylsulphonyl fluoride, 1 mM; pepstatin A, 0.05 mM and leupeptin, 0.2 mM) with gentle shaking. The cell extract was then boiled (10 min) in a ratio of 1:1 with gel loading buffer (Tris, 50 mM; SDS, 10% w/v; glycerol, 10% v/v; 2-mercaptoethanol 10% v/v and bromphenol blue, 2 mg ml^{-1}). Samples were centrifuged at 10,000 *g* for 2 min before being loaded onto a 4% stacking gel/ 7.5% separating gel and subjected to electrophoresis (1.5 h at 125V–200V). The separated proteins were transferred to nitrocellulose (BIORAD; 1 h at 0.3A). After transfer to nitrocellulose, the blot was incubated in blocking solution

(dried minimal-fat milk 25 g and Tween-20 1.25 ml in PBS solutions 500 ml) for 1 h before being primed for 1 h with either rabbit polyclonal antisera raised against purified sheep seminal vesicle COX-1 or rabbit polyclonal antibody raised against purified sheep placental COX-2 (a kind gift from Dr Ian Rodger, Merck Frosst, Point due Claire, Quebec, Canada). The blots were then incubated with a secondary antibody raised in donkey against rabbit, conjugated to horse radish peroxidase and antibody-bound protein visualized by enhanced chemiluminence (ECL, Amersham plc, U.K.). There was no detectable cross reactivity of the COX-1 antibody with COX-2 or the COX-2 antibody with COX-1 (Figure 1a and b).

Northern blot analysis

RNA preparation and Northern blot hybridization analysis
Total RNA from HASM was extracted according to the single step extraction procedure of Chomczynski & Sacchi (1987). Purity and quality were assessed by A_{260}/A_{280} spectrophotometric measurements. Samples were size fractionated on a 1% agarose/formaldehyde gel containing 20 mM morpholinosulphonic acid (MOPS), 5 mM sodium acetate and 1 mM EDTA (pH 7.0) and blotted onto Hybond-N filters (Amersham, U.K.) by capillary action with 20 × SSC (Standard Saline Citrate, 1 × SSC, 0.15 mM NaCl and 0.015 M sodium citrate at pH 7.0).

The blots were prehybridized for 4–6 h at 42°C in buffer consisting of 4 × SSC, 50% formamide, 50 mM Tris-HCl (pH 7.5), 5× Denhardt's solution (0.02% Ficoll/ 0.02% polyvinylpyrrolidone/ 0.02% bovine serum albumin), 0.1% sodium dodecyl sulphate (SDS), 5 mM EDTA and 250 μg ml^{-1} sonicated denatured salmon sperm DNA. The cDNA probe (20–30 ng) were labelled with [^{32}P]-dCTP (3000 Ci mmol^{-1}) (NEN, U.K.) by random priming and added to the prehybridization chambers at a final strength of 1 to 2×10^6 c.p.m. ml^{-1} and incubated for 12–16 h at 42°C. After hybridization, blots were washed to a stringency of 0.1 × SSC, 0.1% SDS at 60°C for 30 min. Blots were then exposed to Kodak OMAT XS film at −70°C with intensifying screens for 1 to 4 days. After exposure, blots were stripped in 50% formamide, 10 mM HaH_2PO_4 for 1 h at 65°C before subsequent rehybridization.

To account for difference in loading or transfer of the mRNA, hybridization with a 1272 bp PstI fragment from rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was performed.

Materials

Tritiated PGE_2, thromboxane B_2 and 6-keto-$PGF_{1\alpha}$ were obtained from Amersham International (Amersham, Bucks, U.K.). IL-1β and IFNγ were purchased from Boehringer Manheim (Boehringer Manheim, Lewes, East Sussex, U.K.) and TNFα from R & D Systems (R & D Systems Europe Ltd., Abingdon, Oxfordshire, U.K.). ECL reagent was obtained from Amersham International (Amersham, Bucks., U.K.). The COX-1 and COX-2 standards were purchased from the Alexis Corporation (Alexis Corporation Ltd., Nottingham, U.K.). Amphotericin B, non essential amino acids, sodium pyruvate were purchased from Life Technologies (Life Technologies Ltd, Paisley, U.K.). All other materials were purchased from Sigma Chemical Company (Poole, U.K.) unless otherwise stated.

Statistical analysis

Results shown are the mean ± s.e.mean from n determinations from HASM cells obtained from 3 or 4 patients. Data were analysed by a Kruskal-Wallis non-parametric ANOVA test followed by a Dunn's test for multiple comparisons. All treatments were compared with control values, $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Results

Characterization of the isoforms of COX present in HASM cells

Untreated HASM cells contained COX-1 protein (Figure 1a) but COX-2 was not detected (Figure 1b). However, after exposure of the cells to the cytokine mixture there was an indution of COX-2 mRNA which apparent 2 h after cytokine treatment, maximal at 12 h and still evident at up to 24 h after treatment (Figure 2a). Furthermore, 24 h after treatment with the cytokine mixture there was a release of PGE_2 (Figure 3), an induction of COX-2 protein with no change in the expression of COX-1 (Figure 1a and b). Furthermore, the release of PGE_2 in response to the cytokine mixture was abolished by the COX-1/COX-2 inhibitor indomethacin (Figure 3). Pretreatment of HASM cells with dexamethasone (1 μM), added 30 min before the addition of the cytokine mixture, almost abolished the induction of COX-2 mRNA and protein (Figures 1b and 2b) and inhibited the release of PGE_2 (Figure 3). Furthermore, cyclohexamide (10 μg ml^{-1}, 30 min before cytokine mixture) inhibited the expression of COX-2 protein and the release of PGE_2 (Figure 3). Alternatively, cyclohexamide alone increased the 'basal' expression of COX-2 mRNA and further increased the cytokine-induced expression of COX-2 mRNA in HASM cells (Figure 2b).

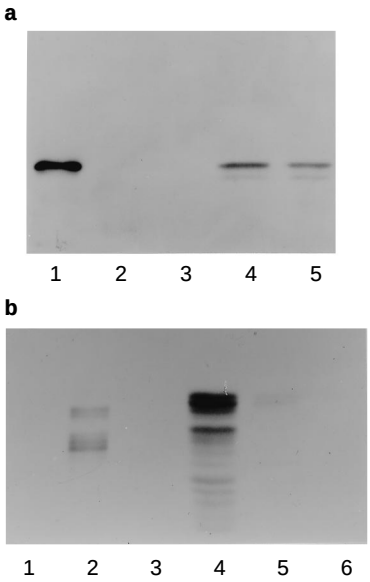

Figure 1 Expression of cyclo-oxygenase-1 (COX-1) and COX-2 protein in HASM cells. (a) A Western blot with a specific antibody to COX-1 which recognizes a band of approximately 70 kDa. Each lane was loaded with 10 μg of protein for samples and 10 ng of protein for the COX-1 and COX-2 standards. Lane 1, COX-1 standard; lane 2, COX-2 standard; lane 3, molecular weight markers; lane 4, extracts from untreated HASM cells; lane 5, extracts from HASM cells treated with a mixture of cytokines (IL-1β, TNFα, IFNγ all at 10 ng ml^{-1} for 24 h). (b) A representative Western blot with a specific antibody to COX-2 which recognises a band of approximately 70 kDa. Each lane was loaded with 10 μg of protein for samples and 10 ng of protein for the COX-1 and COX-2 standards. Lane 1, COX-1 standard; lane 2, COX-2 standard; lane 3, extracts from untreated HASM cells; lane 4, extracts from HASM cells treated with a mixture of cytokines (IL-1β, TNFα, IFNγ all at 10 ng ml^{-1} for 24 h); lane 5, extracts from HASM cells pretreated with dexamethasone (1 μM; for 30 min) before treatment with the mixture of cytokines for 24 h; lane 6, extracts from HASM cells pretreated with cyclohexamide (10 μg ml^{-1}; for 30 min) before treatment with the mixture of cytokines for 24 h.

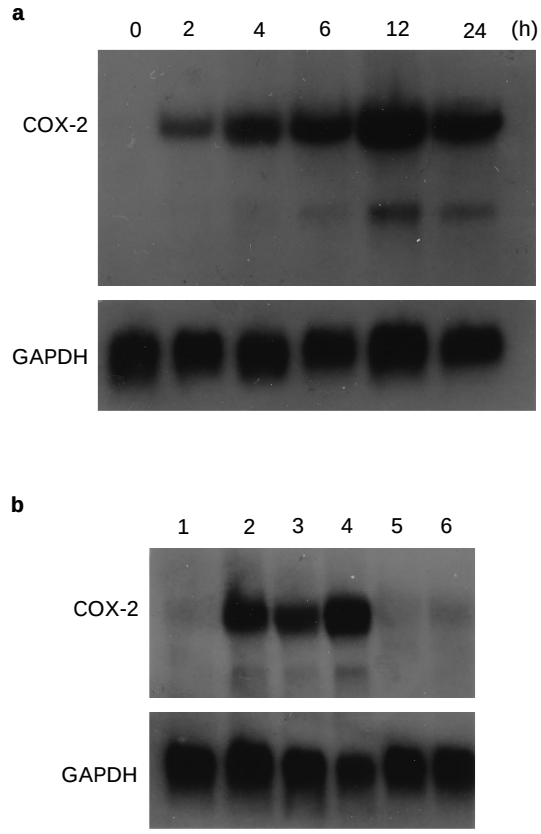

Figure 2 The expression of the 4.5 kb cyclo-oxygenase-2 (COX-2) mRNA in HASM cells. (a) Kinetics of COX-2 mRNA induction in HASM. Cells were treated with the cytokine mixture (IL-1β, TNFα, IFNγ each at 10 ng ml^{-1}) for the time indicated in hours and then harvested for Northern blot analysis. (b) Effect of dexamethasone and cyclohexamide on COX-2 mRNA expression. Cells were treated with, lane 1, vehicle; lane 2, cytokine mixture (IL-1β, TNFα, IFNγ each at 10 ng ml^{-1} for 12 h); lane 3, cyclohexamide alone (10 μg ml^{-1} for 12 h); lane 4, pretreated for 1 h with cyclohexamide (10 μg ml^{-1}) before incubation with the cytokine mixture for 12 h; lane 5, dexamethasone (1 μM for 12 h); lane 6, pretreated with dexamethasone (1 μM for 1 h) before incubation with the cytokine mixture for 12 h. RNA from these samples was then extracted and evaluated for COX-2 mRNA expression by Northern blot analysis. To control for differences in RNA loading, blots were hybridized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which was used as an internal standard.

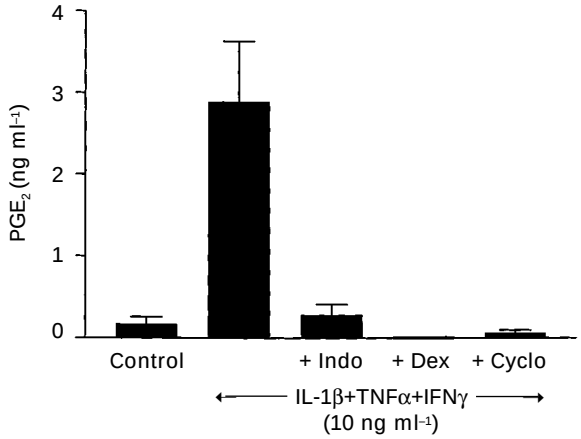

Figure 3 Demonstrates the release of PGE_2 by HASM cells treated with either vehicle (control), or the cytokine mixture (IL-1β, TNFα, IFNγ, all at 10 ng ml^{-1} for 24 h) alone or in the presence of the cytokine mixture with either indomethacin (Indo, 30 μg ml^{-1}) or dexamethasone (Dex, 1 μM) or cyclohexamide (Cyclo, 10 μg ml^{-1}) added 30 min before the addition of cytokines. The data represent the mean$\pm$s.e.mean for 9 determinations from HASM cells from 3 patients.

Release of COX metabolites by HASM cells from endogenous stores of arachidonic acid

Untreated HASM cells released low or undetectable levels of all COX metabolites measured (PGE_2, below level of detection; 6-keto $PFG_{1\alpha}$, 0.258 ± 0.12 ng ml^{-1}; TXB_2 0.162 ± 0.075 ng ml^{-1}, $n=6$) (Figure 4). However, incubation of the cells for 24 h with the cytokine mixture caused the accumulation of PGE_2 (13.67 ± 3.63 ng ml^{-1}, $n=6$), 6-keto $PGF_{1\alpha}$ (8.51 ± 1.56 ng ml^{-1}, $n=6$) and TXB_2 (0.44 ± 0.12 ng ml^{-1}, $n=6$) (Figure 4). In separate experiments, incubation of HASM cells with LPS (0.1 – 10 μg ml^{-1}) or any cytokines individually (0.1 – 100 ng ml^{-1}) did not cause detectable release of PGE_2 (Figure 5). However, the release of PGE_2 was elevated over control levels when HASM cells were incubated with IL-1β together with TNFα for 24 h (each 10 ng ml^{-1}) (control, 0.06 ± 0.041 ng ml^{-1}, $n=12$; IL-1β plus TNFα, 8.719 ± 4.058 ng ml^{-1} $n=12$, $P<0.001$) and after the addition of

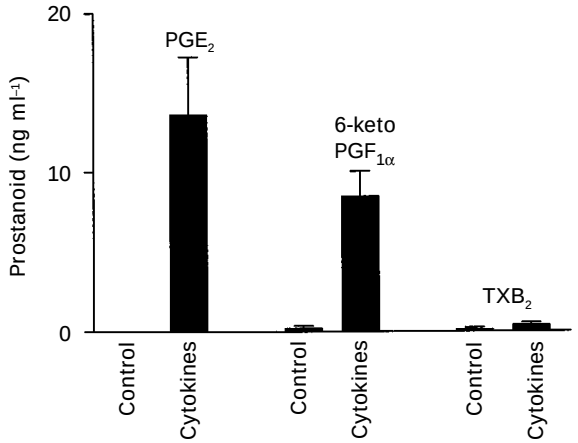

Figure 4 Release of different cyclo-oxygenase (COX) metabolites by HASM in untreated (control) and after treatment with a mixture of cytokines (IL-1β, TNFα, IFNγ all at 10 ng ml^{-1} for 24 h). The data represent the mean$\pm$s.e. for 6 determinations from HASM cells from 2 patients.

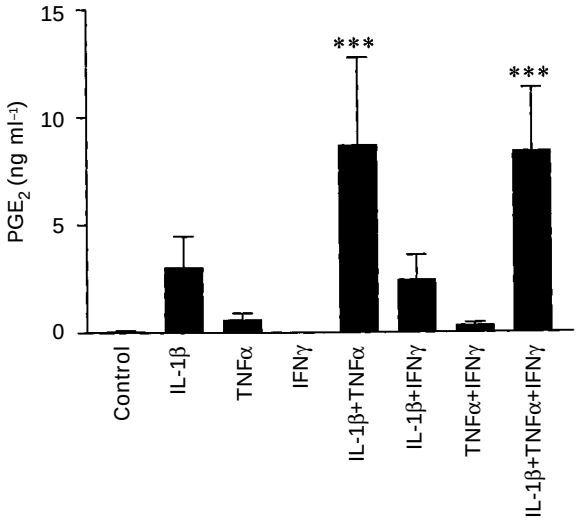

Figure 5 Demonstrates the release of PGE_2 by HASM cells treated with either vehicle (control), IL-1β, TNFα, IFNγ, a mixture of any two cytokines or a mixture of the three cytokines (IL-1β, TNFα and IFNγ) all at 10 ng ml^{-1} for 24 h. The data represent the mean$\pm$s.e.mean for 12 determinations from HASM cells from 4 patients. Significant differences in the release of PGE_2 by vehicle-treated (control) compared to treated cells were calculated by a Kruskal-Wallis Non-parametric ANOVA test followed by Dunn's test for multiple comparisons (***$P<0.001$).

the cytokine mixture (8.412±2.94, $n=12$, $P<0.001$) (Figure 5).

The treatment of HASM cells with the cytokine mixture for 2, 4, 6, 12 and 24 h resulted in a time-dependent release of PGE_2 which was significant at 12 h after the addition of cytokines (Figure 6).

Release of COX metabolites by HASM cells from exogenous stores of arachidonic acid

In HASM cells COX activity (measured in the presence of exogenous arachidonic acid; 30 μM; 15 min) was elevated at 24 h after the addition of IL-1β together with TNFα (each

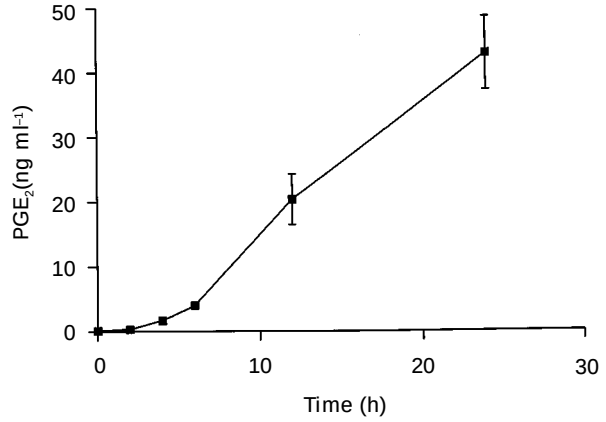

Figure 6 Time-dependent release of PGE_2 from HASM cells after the addition of the cytokine mixture (IL-1β, TNFα, IFNγ all at 10 ng ml^{-1}). Culture medium was removed at 2, 4, 6, 12 and 24 h after the addition of cytokines. No PGE_2 was detected in culture medium removed from cells after the addition of vehicle for the same time points. The data represent 3 determinations from HASM cells obtained from 1 patient.

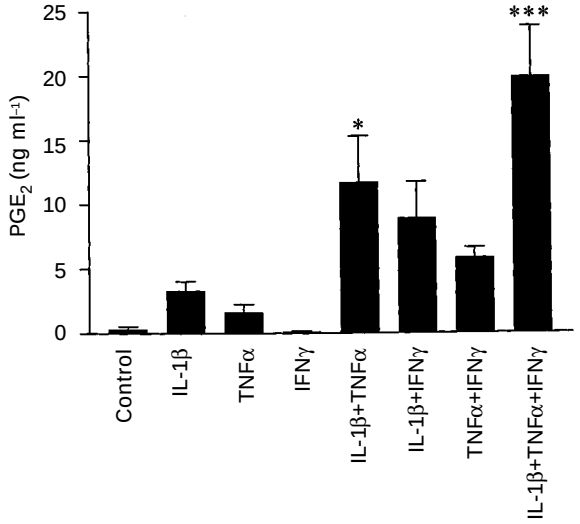

Figure 7 Induction of cyclo-oxygenase (COX) activity (measured in the presence of exogenous arachidonic acid; 30 μM) in HASM cells by different combinations of cytokines. HASM cells were treated with either vehicle (control), IL-1β, TNFα, IFNγ or a mixture of any two cytokines or a mixture of the three cytokines (IL-1β, TNFα, IFNγ) all at 10 ng ml^{-1} for 24 h. The culture medium was then replaced with fresh medium containing arachidonic acid (30 μM) and incubated for 15 min. The formation of PGE_2 by the cells was then measured as an index of COX-2 activity. The data represent the mean±s.e.mean for 6 determinations from HASM cells obtained from 2 patients. Significant differences in the release of PGE_2 by untreated (control) compared to treated cells were calculated by Kruskal-Wallis Non-parametric ANOVA test followed by Dunn's Multiple Comparisons Test (*$P<0.05$, ***$P<0.001$).

10 ng ml^{-1}) (control, 0.352±0.172 ng ml^{-1}, $n=6$; IL-1β plus TNFα, 11.703±3.62 ng ml^{-1}, $n=6$, $P<0.05$) and after the addition of the cytokine mixture (19.899±3.913, $n=6$, $P<0.001$) (Figure 7).

Discussion

Prostanoids, generated under physiological or pathophysiological conditions, influence many aspects of airway smooth muscle function. Thromboxane A_2 has been implicated in the hyperresponsiveness of airway smooth muscle which is associated with allergen challenge (Chung *et al*., 1986) and ozone inhalation (Aizawa *et al*., 1985). Furthermore, thromboxane A_2 has been demonstrated to cause airway hyperresponsiveness to muscarinic agents in asthmatic individuals (Fujimura *et al*., 1986). In contrast, PGE_2 is a potent inhibitor of bronchoconstriction induced by metabisulphite (Pavord *et al*., 1991), allergen (Pavord *et al*., 1993), exercise and distilled water (Passargiklian *et al*., 1976; 1977). However, apart from their actions on airway smooth muscle tone, prostanoids are also known to modulate mitogenesis of airway smooth muscle (Noveral & Grunstein, 1992; Johnson *et al*., 1995). Moreover, as well as eliciting functional responses to prostanoids via activation of cell surface receptors, airway smooth muscle cells themselves can produce prostanoids which may then act in an autocrine fashion (Noveral & Grunstein, 1992; Delamere *et al*., 1994).

It is now established that peptide mediators, such as cytokines and growth factors, orchestrate and perpetuate the chronic inflammation of diseases such as bronchial asthma (Barnes, 1994). Pro-inflammatory cytokines, such as TNFα and IL-1β, regulate the release of several inflammatory mediators by inducing the expression of synthesising enzymes such as COX-2 in airway epithelial cells. (Mitchell *et al*., 1994; Newman *et al*., 1994; Newton *et al*., 1996). We have now extended these findings and have demonstrated that a combination of cytokines (TNFα and IL-1β) stimulate human airway smooth muscle cells to release COX metabolites with no further increase when IFNγ was added to the mixture. In addition, the cytokine mixture increased COX activity and induced the expression of COX-2 mRNA and protein. These results are in agreement with a recent study which demonstrated an increased expression of COX-2 mRNA in rat tracheobronchial smooth muscle cells after exposure of the cells to TNFα and IL-1β (Vadas *et al*., 1996). Low levels of COX-1 protein were detected in untreated cells but these levels were not increased with cytokine treatment. In addition, no detectable release of PGE_2 was found in untreated cells, suggesting that the exaggerated release of prostanoids was not due to an increased expression or activity of the COX-1 protein.

We have found clear differences in cytokine combinations required to reduce COX-2 in human epithelial compared to airway smooth muscle cells. These differences may indicate cell specific signal transduction pathways for COX-2 induction. However, whether these cytokines cause the induction of COX-2 directly or via the release of other mediators has not yet been established. Furthermore, human airway epithelial cells release predominantly PGE_2 when induced to express COX-2 (Mitchell *et al*., 1994), whereas human airway smooth muscle cells release PGE_2 and the stable metabolite of prostacyclin, 6-keto $PGF_{1\alpha}$ in roughly equivalent amounts. The finding that different airway cells release prostanoids in different proportions (Mitchell *et al*., 1994; this paper), suggests that the effects of COX-2 induction in the airway are dependent not only on the type of prostanoid receptors present but also on the population of cells exposed to cytokines.

Furthermore, cyclohexamide, a protein synthesis inhibitor, prevented the induction of COX-2 protein by the cytokine mixture. These results indicate that the enhanced release of prostanoids is dependent on *de novo* transcription and translation. In addition, there was superinduction of COX-2 mRNA in the presence of cyclohexamide, a phenomenon that

has been demonstrated before for other cell types (Lyons-Giordano *et al*., 1993). Moreover, in this study we have demonstrated that the induction of COX-2 mRNA and protein and the release of PGE_2 on cytokine treatment are inhibited by the anti-inflammatory steroid dexamethasone. The inhibitory action of dexamethasone on the expression of pro-inflammatory genes such as inducible nitric oxide synthase has been demonstrated to be linked, in part, to the suppression of nuclear factor (NF)-κB activity (Kleinert *et al*., 1996). Furthermore, the human COX-2 promoter has two putative NF-κB binding sites (Appleby *et al*., 1994) which may indicate that the expression of this inflammatory gene, and the inhibitory action of glucocorticosteroids, are intimately linked to the activity of this transcription factor. Moreover, it is tempting to speculate that the anti-inflammatory action of glucocorticosteroids in inflammatory airway diseases such as bronchial asthma may be associated with the inhibitory action of these compounds on the induction of immediate early genes such as COX-2 and the consequent suppression of prostanoid release at the site of inflammation.

It is evident that prostanoids have wide ranging effects in the airways including modulation of airway and vascular tone, plasma exudation, inflammatory cell recruitment, and activity, cytokine release, mucus secretion and cholinergic and sensory nerve function. However, it is still unknown whether the increased expression of COX-2 in human airway smooth muscle cells and the exaggerated production of PGE_2 and prostacyclin would be deleterious or beneficial for airway function in inflammatory diseases such as asthma. An alternative hypothesis to COX-2 expression being a pro-inflammatory event could be that prostanoid production by COX-2 is a natural defence mechanism of the airways to inflammation. In fact, the induction of COX-2 in human airway smooth muscle could theoretically shunt arachidonic acid away from the production of constrictor leukotrienes towards the production of dilator prostaglandins such as PGE_2 and prostacyclin. In this way inhibition of prostanoid production with non-steroidal anti-inflammatory drugs in a population of asthmatics that express larger amounts of cyclo-oxygenase (eg. COX-2) could theorectically lead to the phenomenon known as 'aspirin-sensitive' (Dahlén & Dahlén, 1995).

In conclusion, the data presented here demonstrate COX-2 induction in human airway smooth muscle cells in response to stimulation with pro-inflammatory cytokines. This enhanced release of prostanoids from human airway smooth muscle cells reveals a novel role for this cell as an inflammatory cell in addition to its contractile function. This observation is consistent with recent data demonstrating the release of the chemokines RANTES (John *et al*., 1996) and IL-8 (Watson *et al*., 1996) from human airway smooth muscle cells on cytokine stimulation. Therefore, these results would seem to indicate that airway smooth muscle cells contribute to airway inflammation by interacting with cytokines to produce various mediators and chemoattractants which can evoke cellular infiltration and therefore perpetuate the inflammatory response.

M.G.B. and S.J.H. are supported by Wellcome Post-doctoral Fellowships, J.A.M. is supported by the British Heart Foundation, M.A.S. by the British Lung Foundation and E.-B.H. by a grant from the European Union. The authors would like to thank Dr Robert Newton for his help and advice with the COX-2 Northern blots.

References

AIZAWA, H., CHUNG, K.F., LEIKAUF, G.D., UEKI, I., BETHEL, R.A., O'BYRNE, P.M., HIVOSE, T. & NADEL, J.A. (1985). Significance of thromboxane generation in ozone-induced airway hyperresponsiveness in dogs. *J. Appl. Physiol.*, **59,** 1918 – 1923.

APPLEBY, S.B., RISTIMAKI, A., NEILSON, K., NARKO, K. & HLA, T. (1994). Structure of the human cyclo-oxygenase-2 gene. *Biochem. J.*, **302,** 723 – 727.

BARNES, P.J. (1994). Cytokines as mediators of chronic asthma. *Am. J. Respir. Crit. Care Med.*, **150,** S42 – S49.

CHAN, C.-C., BOYCE, S., BRIDEAU, C., FORD-HUTCHINSON, A.W., GORDON, R., GUAY, D., HILL, R.G., LI, C.-S., MANCINI, J., PENNETON, M., PRASIT, P., RASORI, R., RIENDEAU, D., ROY, P., TAGARI, P., VICKERS, P., WONG, E. & RODGER, I.W. (1995). Pharmacology of a selective cyclooxygenase-2 inhibitor, L-745, 337: a novel nonsteroidal anti-inflammatory agent with an ulcerogenic sparing effect in rat and nonhuman primate stomach. *J. Pharmacol. Exp. Ther.*, **274,** 1531 – 1537.

CHOMCZYNSKI, P. & SACCHI, N. (1987). Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162,** 156 – 159.

CHUNG, K.F., AIZAWA, H., BECKER, A.B., FRICK, O., GOLD, W.M. & NADEL, J.A. (1986). Inhibition of antigen-induced airway hyperresponsiveness by a thromboxane synthetase inhibitor (OKY 046) in allergic dogs. *Am. Rev. Respir. Dis.*, **134,** 258 – 261.

DAHLÉN, B. & DAHLÉN, S.-E. (1995). Leukotrienes as mediators of airway obstruction and inflammation in asthma. *Clin. Exp. Allergy*, **25,** 50 – 54.

DELAMERE, F., HOLLAND, E., PATEL, S., BENNETT, J., PAVORD, I. & KNOX, A. (1994). Production of PGE_2 by cultured bovine airways smooth muscle cells and its inhibition by cyclo-oxygenase inhibitors. *Br.J. Pharmacol.*, **111,** 983 – 988.

FUJIMURA, M.F., SASAKI, F., NAKATSUMI, Y., TAKAHASHI, Y., HIFUMI, S., TAGA, K., MIFUNE, J.I., TANAKA, T. & MATSUDA, T. (1986). Effects of a thromboxane synthetase (OKY-046) and a lipoxygenase inhibitor (AA-861) on bronchial responsiveness to acetylcholine in asthmatic subjects. *Thorax*, **41,** 955 – 959.

HAMBERG, M., SVENSSON, J. & SAMUELSSON, B. (1974). Novel transformation of arachidonic acid in human platelets. *Proc. Natl. Acad. Sci. U.S.A.*, **71,** 3400 – 3404.

HEMPEL, S., MONICK, M.M. & HUNNINGHAKE, G.W. (1994). Lipopolysaccharide induces prostaglandin H synthase-2 protein and mRNA in human alveolar macrophages and blood monocytes. *J. Clin. Invest.*, **93,** 391 – 396.

HIRST, S.J., BARNES, P.J. & TWORT, C.H.C. (1992). Quantifying proliferation of cultured human and rabbit airway smooth muscle cells in response to serum and platelet derived growth factor. *Am. J. Respir. Cell Molec. Biol.*, **7,** 574 – 581.

JOHN, M., ROBICHAUD, A., HIRST, S., JOSE, P., BERKMAN, N., WITT, C., BARNES, P.J. & CHUNG, K.F. (1996). Human airway smooth muscle cells (HASMC) express and release rantes: regulation by TH-2 cytokines and corticosteroids. *Am. J. Respi. Crit. Care Med.*, **153,** A253.

JOHNSON, P.R.A., CAREY, D., ARMOUR, C.L. & BLACK, J.L. (1995). PGE_2 and heparin are antiproliferative mediators for human airway smooth muscle cells in culture. *Am. J. Physiol.*, **13,** L514 – L519.

KLEINERT, H., EUCHENHOFER, C., IHRIG-BIEDERT, I. & FÖRSTERMANN, U. (1996). Glucocorticoids inhibit the induction of nitric oxide synthase II by down-regulating cytokine-induced activity of transcription factor nuclear factor-κB. *Mol. Pharmacol.*, **49,** 15 – 21.

LEE, S.H., SOYOOLA, E., CHANMUGAM, P., HART, S., SUN, W., ZHONG, H., LIOU, S., SIMMONS, D. & HWANG, D. (1992). Selective expression of mitogen-inducible cyclo-oxygenase in macrophages stimulated with lipopolysaccharide. *J. Biol. Chem.*, **267,** 25934 – 25938.

LYONS-GIORDANO, B., PRATTA, M.A., GALBRAITH, W., DAVIS, G.L. & ARNER, E.C. (1993). Interleukin-1 differentially modulates chondrocyte expression of cyclooxygense-2 and phospholipase A_2. *Exp. Cell Res.*, **206,** 58 – 62.

MAIER, J.A., HLA, T. & MACIAG, T. (1990). Cyclo-oxygenase is an immediate early gene induced by interleukin-1 in human endothelial cells. *J. Biol. Chem.*, **265,** 10805 – 10808.

MITCHELL, J.A., AKARASEREENONT, P., THIEMERMANN, C., FLOWER, R.J. & VANE, J.R. (1993). Selectivity of nonsteroid anti-inflammatory drugs as inhibitors of constitutive and inducible cyclo-oxygenase. *Proc. Natl. Acad. Sci. U.S.A.*, **90,** 11693 – 11697.

MITCHELL, J.A., BELVISI, M.G., AKARESEREENONT, P., ROBBINS, R.A., KWON, O.-J., CROXTALL, J., BARNES, P.J. & VANE, J.R. (1994). Induction of cyclo-oxygenase-2 by cytokines in human pulmonary epithelial cells: regulation by dexamethasone. *Br. J. Pharmacol.*, **113,** 1008 – 1014.

MITCHELL, J.A., LARKIN, S. & WILLIAMS, T.J. (1995). Cyclo-oxygenase-2: regulation and relevance in inflammation. *Biochem. Pharmacol.*, **50,** 1535 – 1542.

NEWMAN, S.P., FLOWER, R.J. & CROXTALL, J.D. (1994). Dexamethasone suppression of IL-1β-induced cyclooxygenase 2 expression is not mediated by lipocortin-1 in A549 cells. *Biochem. Biophys. Res. Commun.*, **202,** 931 – 939.

NEWTON, R., KUITERT, L.M., SLATER, D.M., ADCOCK, I.M. & BARNES, P.J. (1996). Cytokine induction of cytosolic phospholipase A_2 and cyclooxygenase-2 mRNA is suppressed by glucocorticoids in human epithelial cells. *Life Sci.*, **60**, 67 – 77.

NOVERAL, J.P. & GRUNSTEIN, M.M. (1992). Role and mechanism of thromboxane-induced proliferation of cultured airway smooth muscle cells. *Am. J. Physiol.*, **263,** L555 – L561.

O'SULLIVAN, M.G., HUGGINS, E.M., MEADE, E.A., DEWITT, D.L. & MCCALL, C.E. (1992). Lipopolysaccharide induces prostaglandin H synthase-2 in alveolar macrophages. *Biochem. Biophys. Res. Commun.*, **187,** 1123 – 1127.

PASSARGIKLIAN, M., BIANCO, S. & ALLEGRA, L. (1976). Clinical, functional and pathogenetic aspects of bronchial reactivity to prostaglandlins $F_{2\alpha}$, E_1 and E_2. *Adv. Prostaglandin Thromboxane Res.*, **1,** 461 – 475.

PASSARGIKLIAN, M., BIANCO, S., ALLEGRA, L., MOAVERO, N.E., PETRIGINI, G., ROBUSCHI, M. & GRUGNI, A. (1977). Aspects of bronchial reactivity to prostaglandins, and aspirin in asthmatic patients. *Respiration*, **34,** 79 – 91.

PAVORD, I.D., WISNIEWSKI, A.L., MATHUR, R., WAHEDNA, I., KNOX, A.J. & TATTERSFIELD, A.E. (1991). Effect of inhaled PGE_2 on bronchial reactivity to sodium metabisulphite, and methacholine in patients with asthma. *Thorax*, **46,** 633 – 637.

PAVORD, I.D., WONG, C., WILLIAMS, J. & TATTERSFIELD, A.E. (1993). Effect of inhaled prostaglandin E2 on allergen-induced asthma. *Am. Rev. Respir. Dis.*, **148,** 87 – 90.

SALMON, J.A. (1978). A radioimmunoassay for 6-keto-prostaglandins $F_{1\alpha}$. *Prostaglandins*, **5,** 383 – 397.

SAUNDERS, M.A., MITCHELL, J.A., HIRST, S.J., WILLIAMS, T.J., YACOUB, M.H., BARNES, P.J. & BELVISI, M.G. (1996). Characterisation of cyclo-oxygenase-2 induction in human airways smooth muscle cells. *Br. J. Pharmacol.*, **117,** 82P.

TWORT, C.H.C. & VAN BREEMAN, C. (1988). Human airway smooth muscle in culture. *Tissue Cell*, **20,** 339 – 344.

VADAS, P., STEFANSKI, E., WLOCH, M., GROUIX, B., VAN DEN BOSCH, H. & KENNEDY, B. (1996). Secretory non-pancreatic phospholipase A_2 and cyclooxygenase-2 expression by tracheobronchial smooth muscle cells. *Eur. J. Biochem.*, **235,** 557 – 563.

VANE, J.R., MITCHELL, J.A., APPLETON, I., TOMLINSON, A., BISHOP-BAILEY, D.A., CROXTALL, J. & WILLOUGHBY, D. (1994). Inducible isoforms of cyclo-oxygenase and nitric oxide synthase in inflammation. *Proc. Natl. Acad. Sci. U.S.A.*, **91,** 2046 – 2050.

WATSON, M.L., GRIX, S.P., JORDAN, N.J., PLACE, G.A., DODD, S., LEITHEAD, J., POLL, C.T. & WESTWICK, J. (1996). IL-8 production by human cultured airway smooth muscle. *Br. J. Pharmacol.*, **119,** 47P.

XIE, W.L., CHIPMAN, J.G., ROBERTSON, D.L., ERIKSON, R.L. & SIMMONS, D.L. (1991). Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc. Natl. Acad. Sci. U.S.A.*, **88,** 2692 – 2696.

(Received August 29, 1996
Revised October 25, 1996
Accepted November 8, 1996)