Production of PGE$_2$ by bovine cultured airway smooth muscle cells and its inhibition by cyclo-oxygenase inhibitors

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1 Prostaglandin E$_2$ (PGE$_2$) is thought to be an important inhibitory modulator of inflammatory processes in the airway. It inhibits inflammatory cell function and cholinergic neurotransmission in vitro and roles have been postulated in vivo in refractoriness and in the mechanism of action of the diuretic agent, frusemide.

2 The production of PGE$_2$ by bovine cultured airway smooth muscle cells has been studied under a range of conditions. The effects of cyclo-oxygenase inhibitors (flurbiprofen, indomethacin, acetyl salicylic acid) on serum-induced production of PGE$_2$ were assessed over a range of concentrations ($10^{-7}$–$10^{-5}$ M).

3 Serum-stimulated production of PGE$_2$ in control wells ranged from 350 to 800 ng PGE$_2$ ml$^{-1}$ in cells from different animals. All three cyclo-oxygenase inhibitors inhibited PGE$_2$ production with an order of potency, flurbiprofen > indomethacin > acetyl salicylic acid. Log IC$_{50}$ values were -6.24 for flurbiprofen, -5.23 for indomethacin and -3.50 for acetyl salicylic acid.

4 PGE$_2$ production was stimulated by arachidonic acid ($10^{-5}$ M) or addition of the proinflammatory mediator, bradykinin ($10^{-7}$–$10^{-5}$ M).

5 Incubation of cells for 24 h with 5 bromo deoxyuridine (BRDU) ($10^{-4}$ M) to prevent DNA synthesis did not alter PGE$_2$ production in response to serum, suggesting that it was not a function of proliferation per se.

6 Our study suggests that airway smooth muscle may be an important source of PGE$_2$. Production of PGE$_2$ may be a novel feedback mechanism whereby airway smooth muscle cells can negatively modulate airways inflammation. The differing potencies of the cyclo-oxygenase inhibitors may explain the contrasting effect of these drugs in recent studies in asthma.

Keywords: PGE$_2$; airway smooth muscle; indomethacin; flurbiprofen; acetyl salicylic acid; bradykinin

Introduction

Prostaglandin E$_2$ (PGE$_2$) is thought to be an important inhibitory prostaglandin in airway tissue which has potent effects both in vitro and in vivo. In vitro, PGE$_2$ inhibits acetylcholine release from parasympathetic nerve endings in human airway smooth muscle (Ito et al., 1990). It inhibits release of mediators from human lung mast cells (Peters et al., 1982) and inhibits cellular responses in other inflammatory cells such as eosinophils (Giembycz et al., 1990), macrophages (Christman & Christman, 1990) and T-lymphocytes (Minakuchi et al., 1990). In asthmatic subjects in vivo, PGE$_2$ is a potent inhibitor of bronchostenosis induction by metabisulphite (Pavord et al., 1991), allergen (Pavord et al., 1992a), exercise and ultrasonically nebulised distilled water (Pasargiklian et al., 1976; 1977). A role for PGE$_2$ has also been postulated in the refractoriness to bronchoconstrictor challenges which occurs in asthma (Pavord et al., 1992a) and in the effect of the diuretic drug, frusemide, which protects against several bronchoconstrictor challenges in asthma (Pavord et al., 1992a).

It is not known which of the many types of airway cells are the most important in PGE$_2$ production as this has not been studied in detail with the exception of the epithelial cell. Airway smooth muscle has been relatively ignored as a possible site of production. A few studies in lung homogenates (Masamoto & Masuda, 1976) or whole airway preparations (Haye-Legrand et al., 1986) have documented production of PGE$_2$, suggesting that airway smooth muscle is capable of PGE$_2$ synthesis but these preparations contain heterogenous populations of airway cells. As smooth muscle is an important source of prostaglandins in other organs (Chamley-Campbell et al., 1979) it would seem likely that airway smooth muscle cells may also be an important source of PGE$_2$. In one preliminary report on dog tracheal smooth muscle strips, PGE$_2$ was produced in response to histamine, suggesting that airway smooth muscle is capable of PGE$_2$ synthesis (Anderson et al., 1980). One method of studying PGE$_2$ production by airway smooth muscle more closely is to study cultured cells.

Cyclo-oxygenase inhibitors have been recently shown to have conflicting effects in asthma (Robuschi et al., 1992; Bianco et al., 1992; Pavord et al., 1992b; Polosa et al., 1992; O'Connor et al., 1993). In view of this and the potential importance of PGE$_2$ in the airway, we studied the production of PGE$_2$ by cultures of bovine airway smooth muscle cells and its inhibition by flurbiprofen, indomethacin and acetyl salicylic acid.

Methods

Tissue preparation

Bovine trachea was obtained from the local abattoir and bathed immediately in ice cold Krebs-Henseleit solution. The tracheal muscle was then dissected free of epithelium and connective tissue. The muscle was minced with a McIlwain tissue chopper (Brinkmann Instruments, Westbury, New York, U.S.A.) and subjected to an enzymatic digestion procedure (Felbel et al., 1988; Kamm et al., 1989). The chopped tissue was incubated in 5 ml Krebs-Ringer-HEPES solution (KRH) without enzymes and shaken in a waterbath at 37°C for 30 min. The tissue was then incubated in 5 ml Krebs-Ringer-HEPES solution (KRH), containing 3.5 mg type IV collagenase, 5 mg soya bean trypsin inhibitor and 5 mM CaCl$_2$ at 37°C in a shaking water bath for 30 min. This

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procedure was then repeated twice more. The slurry was filtered, the cells were centrifuged, resuspended in Dulbecco’s modified eagles medium (DMEM), counted and then plated out in several 175 cm² flasks. Cells were grown to confluence in DMEM + 10% fetal calf serum (FCS) containing penicillin/streptomycin/amphotericin B and L-glutamine (concentrations given below). Once confluent, cells were trypsinized with 0.25% trypsin, 0.02% EDTA and either passed immediately or resuspended in 90% FCS + 10% dimethyl sulfoxide, frozen in liquid nitrogen and stored until required. Cells were thawed prior to use and plated at a density of 0.2 x 10⁶ cells/well in 12 well plates containing DMEM + 10% fetal calf serum + penicillin/streptomycin/amphotericin B + L-glutamine.

Cell counting

Cells were removed from plates by incubation with 0.25% trypsin, 0.02% EDTA for 8–10 min. Cells were counted in a haemocytometer and viability assessed by trypan blue exclusion.

Radioimmunoassay for PGE₂

PGE₂ was measured by radioimmunoassay (Hawthorne et al., 1991). Assays were performed in triplicate and expressed as pg ml⁻¹. Samples and standards were incubated overnight with assay antibody [³H]-PGE₂ (Amersham P313) (up to 5-bromo deoxyuridine (FITC) conjugated antiserum (Dako F313) (up to 1 in 8 dilution) before being washed and viewed by fluorescent microscopy.

Drugs

Indomethacin, acetyl salicylic acid and flurbiprofen were obtained from Sigma Chemicals, Poole, UK. Rabbit PGE₂ antiserum, PGE₂ standard, type IV collagenase, protease, DMEM, L-glutamine, penicillin/streptomycin, arachidonic acid, bradykinin, 5 bromo deoxyuridine, acetic acid, insulin, transferrin and soya bean trypsin inhibitor were obtained from Sigma Chemicals, Poole, U.K. Amphotericin was obtained from ICN Flow, High Wycombe, U.K. Bovine foetal calf serum was obtained from Seralab, Crawley Down, U.K.

Buffers: Krebs-Henseleit solution had the following composition (mmol l⁻¹): Na⁺Cl⁻ 118, K⁺Cl⁻ 4.7, Mg²⁺SO₄²⁻ 1.2, Na⁺H₂PO₄ 1.2, Ca²⁺Cl⁻ 2.0, L-glutamine, HCO₃⁻ 25, glucose 11.1, pH 7.4 when gassed with 95% O₂, 5% CO₂.

Krebs-Ringer-HEPES was of the following composition (mmol l⁻¹): Na⁺Cl⁻ 105, K⁺Cl⁻ 5, Ca²⁺Cl⁻ 2, K⁺H₂PO₄ 1, Mg²⁺SO₄²⁻ 1, glucose 14, HEPES 25 and phenol red 10 µg ml⁻¹, pH 7.4.

Culture medium

Penicillin/streptomycin solution (5000 u penicillin + 5 mg streptomycin ml⁻¹) 1 ml + 0.5 ml amphotericin (250 µg ml⁻¹) + 1 ml L-glutamine (200 mM) was added to 50 ml of DMEM before use.

Protocols

Cells were used on the first passage and plated at a concentration of 0.2 x 10⁶ cells per well in 12 well culture plates containing DMEM + 10% foetal calf serum (FCS). Initial experiments were performed to determine the day in culture that PGE₂ production was maximal by counting cells and measuring PGE₂ production on days 5, 6, 7 and 8 of culture. PGE₂ production had plateaued by days 6 and 7 and subsequent experiments were performed to look at the effects of cyclo-oxygenase at this time. The time course of PGE₂ production in 10% FCS over 24 h was studied. For these experiments medium was changed on Day 6 and the fresh medium subsequently removed for measurement of PGE₂ at 2, 6 and 24 h from separate wells.

Experiments were performed to look at the effects of different cyclo-oxygenase inhibitors in tissue from four different animals, assays being performed in triplicate for each animal. Indomethacin, acetyl salicylic acid or flurbiprofen were added over a concentration range 10⁻³ - 10⁻⁵ M on day 5 to separate wells of cells. On day 6, after 24 h incubation in each inhibitor, the supernatant from each well was collected, PGE₂ was measured and cells were trypsinized and counted.

Additional experiments were performed to determine the effects of bradykinin, arachidonic acid and foetal calf serum in quiescent cells. In these experiments cells were grown to confluence and growth arrested for 24 h by incubation in serum-free DMEM + insulin 6 µg ml⁻¹ + transferrin 5 µg ml⁻¹ + ascorbic acid 35 µg ml⁻¹ prior to the addition of each agent. PGE₂ production was measured over 6 h.

In order to determine if production of PGE₂ was a function of cell proliferation we performed experiments where cells were incubated in 10⁻⁴ M 5-bromo deoxyuridine (BrdU) for 24 h prior to the addition of 10% FCS and measurement of PGE₂.

Results

Cells grown in the manner described reached confluence after 5–6 days. showed the characteristic appearance of smooth muscle cells in culture with a hill and valley appearance when confluent and stained positively for smooth muscle alpha actin in contrast to fibroblasts grown from connective tissue implants (Figure 1). The initial experiments showed that airway smooth muscle cells cultured in 10% FCS produce large quantities of PGE₂ and that this was maximal after 6–7 days of culture (Table 1). PGE₂ production increased progressively over a 24 h period (Figure 2). Serum-stimulated PGE₂ production in control wells ranged from 350 to 800 ng per well. PGE₂ production was inhibited by all three cyclo-oxygenase inhibitors in a concentration-dependent manner. The concentrations used in
Produced a acetyl production by Flurbiprofen ves. stimulated over of control altered tors 96% and 500 times.

This illustrates the value. Thus was not acid. None of the three inhibitors shown as concentration-response curves. Flurbiprofen was ten times more potent than indomethacin and 500 times more potent than acetyl salicylic acid. Flurbiprofen at 3 × 10^-5 M inhibited PGE_2 production by 97%, indomethacin at 10^-4 M inhibited PGE_2 production by 96% and acetyl salicylic acid at 3 × 10^-3 M inhibited PGE_2 production by 93%. Log IC_{50} values were -6.24 ± 0.1 for flurbiprofen, -5.23 ± 0.1 for indomethacin and -3.50 ± 0.3 for acetyl salicylic acid. None of the cyclo-oxygenase inhibitors altered cell counts significantly at any concentration.

Incubation of cells in serum-free medium for 24 h produced a lower basal level of PGE_2 production. This was stimulated over 6 h by 10^-5 M arachidonic acid to 148 ± 17% of control wells (P<0.05, n = 4 experiments each performed in triplicate). Addition of 10% FCS produced a marked increase in PGE_2 production over 6 h compared to serum-free alone (P<0.05). PGE_2 production over 6 h was 10.4 ± 4 ng per well in serum-free medium and 190 ± 78 ng per well in 10% FCS (n = 4 experiments each performed in triplicate).

Arachidonic acid (10^-3 M) increased PGE_2 production to 148 ± 17% of control values (P<0.05, n = 4 experiments each performed in triplicate). Bradykinin caused a concentration-related increase in PGE_2 production. Bradykinin (10^-6 M) increased PGE_2 production to 239 ± 80% of control values (n = 4 experiments each performed in triplicate) (P<0.05, Figure 4). Incubation of cells with 10^-4 M BrDU for

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**Table 1** Cell counts and prostaglandin E_2 (PGE_2) production from bovine airway smooth muscle cells in culture, day 4-7

<table>
<thead>
<tr>
<th>Day</th>
<th>Mean cell count (x 10^6 cells ml^-1)</th>
<th>Mean PGE_2 prod (ng ml^-1)</th>
<th>PGE_2 prod (ng per 10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>6.2 ± 0.5</td>
<td>174 ± 5.2</td>
<td>28.1</td>
</tr>
<tr>
<td>5</td>
<td>24.3 ± 2.7</td>
<td>327 ± 33.7</td>
<td>13.5</td>
</tr>
<tr>
<td>6</td>
<td>16.3 ± 0.9</td>
<td>747 ± 24.0</td>
<td>45.8</td>
</tr>
<tr>
<td>7</td>
<td>17.0 ± 0.4</td>
<td>837 ± 14.1</td>
<td>49.2</td>
</tr>
</tbody>
</table>

Values shown are mean ± s.e.mean, n = 3.
Discussion

The aim of the study was to measure PGE₂ production by airway smooth muscle cells under different conditions and to study its inhibition by three cyclo-oxygenase inhibitors. We performed experiments on bovine cultured airway smooth muscle cells in vitro. The advantage of using cell culture for these studies is that lipid metabolism can be readily studied and it is easier to manipulate the system experimentally than in studies in tissue slices or whole animals. It also allows the study of individual cell types such as airway smooth muscle without confounding effects due to epithelial and inflammatory cells. We have been able to culture bovine airway smooth muscle cells readily from enzyme digested tissue strips and have confirmed that these cells stain positively for the smooth muscle contractile protein alpha actin unlike confluent cultures of fibroblasts grown from connective tissue explants.

We measured PGE₂ by radioimmunoassay. This is a convenient and reliable technique. The monoclonal antibody used, however, does not discriminate between PGE₁ and PGE₂. However, PGE₁ is produced in minute amounts relative to PGE₂ and can be measured in the PGE₂. We did not measure other arachidonic acid metabolites as h.p.l.c. studies have shown that PGE₂ is by far the most abundant arachidonic acid metabolite of airway smooth muscle (Tanaka et al., 1993).

Ours is the first study to examine PGE₂ production in detail in cultured airway smooth muscle cells. There have, however, been studies measuring PGE₂ production in whole airway tissue preparations which have contained airway smooth muscle in addition to other cell types (Masumoto & Masuda, 1976; Haye-Legrand et al., 1986) and one previous study measuring PGE₂ in canine tracheal strips (Anderson, 1980). However, studies in whole tissue preparations cannot identify the cell type responsible for prostaglandin production.

PGE₂ was stimulated over basal levels in our experiments by addition of arachidonic acid, the substrate for phospholipase A₂. As bradykinin has been shown to increase PGE₂ production in other cell types (White et al., 1992) and is thought to be an important proinflammatory mediator in asthma, we studied the effect of bradykinin on PGE₂ production. Bradykinin caused a concentration-related increase in PGE₂. The finding that proinflammatory mediators such as bradykinin increase PGE₂ is important for several reasons. As PGE₂ is an inhibitory prostaglandin, the PGE₂ released may be involved in a negative feedback mechanism reducing the airway from excessive bronchoconstriction. Proinflammatory mediators such as histamine and bradykinin have also been shown to stimulate PGE₂ production from canine tracheal strips (Anderson et al., 1980) and in human cultured tracheal epithelial cells (Churchill et al., 1989) respectively. Any PGE₂ release by these mediators might feed back to inhibit inflammation. In addition, PGE₂ can reduce or inhibit the airway from excessive bronchoconstriction. Proinflammatory mediators such as histamine and bradykinin have also been shown to stimulate PGE₂ production from canine tracheal strips (Anderson et al., 1980) and in human cultured tracheal epithelial cells (Churchill et al., 1989) respectively. Any PGE₂ release by these mediators might feed back to inhibit inflammation. In addition, PGE₂ can reduce or inhibit the airway from excessive bronchoconstriction. Proinflammatory mediators such as histamine and bradykinin have also been shown to stimulate PGE₂ production from canine tracheal strips (Anderson et al., 1980) and in human cultured tracheal epithelial cells (Churchill et al., 1989) respectively. Any PGE₂ release by these mediators might feed back to inhibit inflammation. In addition, PGE₂ can reduce or inhibit the airway from excessive bronchoconstriction.

In addition to quantifying PGE₂ production by airway smooth muscle cells, we were interested in looking at the contrasting effects of three inhibitors of cyclo-oxygenase. PGE₂ production by cultured bovine airway smooth muscle cells in vitro. The advantage of using cell culture for these studies is that lipid metabolism can be readily studied and it is easier to manipulate the system experimentally than in studies in tissue slices or whole animals. It also allows the study of individual cell types such as airway smooth muscle without confounding effects due to epithelial and inflammatory cells. We have been able to culture bovine airway smooth muscle cells readily from enzyme digested tissue strips and have confirmed that these cells stain positively for the smooth muscle contractile protein alpha actin unlike confluent cultures of fibroblasts grown from connective tissue explants.

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the three prostaglandin synthesis inhibitors might explain their contrasting effects in asthma. It has been known for years that some asthmatic subjects respond to orally administered acetyl salicylic acid and similar drugs by bronchoconstriction (Szczeklik, 1976). However, recently Bianco and colleagues (1992) found that inhaling acetyl salicylic acid had no protective effects in asthma and that its effects are additive with the diuretic agent frusemide. (Robuschi et al., 1992; Bianco et al., 1992). In contrast, oral indomethacin inhibits the protective effect of frusemide on exercise-induced bronchoconstriction (Pavord et al., 1992a) giving rise to the hypothesis that frusemide may be acting by producing PGE2. One flurbiprofen did not inhibit the protective effect of frusemide on exercise-induced bronchoconstriction in asthma (O’Connor et al., 1993) but did inhibit the protective effect of frusemide on methacholine-induced bronchoconstriction in normal subjects (Polosa et al., 1992). In our study acetyl salicylic acid was a relatively poor inhibitor of prostaglandin synthesis and it is therefore unlikely that the beneficial reported effects of inhaled aspirin in asthma are due to inhibition of prostaglandin synthesis. However, the potent effects of indomethacin on prostaglandin synthesis in airway smooth muscle cells in vitro would be consistent with its inhibitory effects on the action of frusemide in vivo if frusemide were acting through prostanooid mediated pathways.

The other implications of our study may be in aspirin-induced asthma which occurs in a small proportion of asthma patients. Several theories have been put forward to explain this, including an imbalance of constrictor and dilator prostaglandins or shunting of arachidonic acid metabolism or leukotriene synthesis (Szczeklik, 1976). The low potency of acetyl salicylic acid at inhibiting PGE2 production would be consistent with the lack of effect of oral aspirin in the majority of patients. It may be that aspirin-sensitive asthmatics are more sensitive to the effects of aspirin on prostaglandin synthesis in asthma.

In conclusion we have demonstrated that bovine airway smooth muscle cells produce large quantities of PGE2 and that this can be inhibited by cyclo-oxygenase inhibitors. PGE2 produced by airway smooth muscle may be an important negative modulator of inflammation in the airways.

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References


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