Selectivity of cyclo-oxygenase inhibitors in human pulmonary epithelial and smooth muscle cells

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ABSTRACT: Cyclo-oxygenase (COX) inhibitors may have a role in reducing inflammation in asthma and other pulmonary diseases. COX inhibitors have different selectivities for the two COX isoenzymes (COX 1 and COX 2) which vary between purified enzyme and intact cell preparations. The relative selectivity of COX inhibitors has not been studied in human airway cells.

A number of COX inhibitors in cultured human airway cells were compared which exclusively express either COX 1 (primary degree cultured human airway smooth muscle (HASM) cells) or COX 2 (A549 pulmonary epithelial cell-line) as measured by Western blotting. COX activity was assayed by prostaglandin (PG)E2 production following 30 min incubation with 5 mM arachidonic acid.

COX activity in both cell types was similar; HASM cells 92.2±12.1 ng PGE2·mg⁻¹ protein, A549 cells 87.7±24.4 ng PGE2·mg⁻¹ protein. In HASM cells the median inhibitory concentration (IC₅₀) was >10⁻⁵ M for nimesulide, 3.2×10⁻⁴ M for N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulphonamide (NS398), 1.8×10⁻⁶ M for flurbiprofen, 6.7×10⁻⁷ M for indomethacin and >10⁻⁹ M for aspirin. In A549 cells the IC₅₀ was 1.8×10⁻⁶ M for nimesulide, 4.1×10⁻⁷ M for NS398, 6.2×10⁻⁸ M for flurbiprofen, 1.3×10⁻⁸ M for indomethacin and >10⁻¹⁰ M for aspirin. Sodium valerate had no effect in either HASM or A549 cells. The COX 2:COX 1 selectivity ratio (COX 2 IC₅₀/COX 1 IC₅₀) was <0.0001 for nimesulide, 0.001 for NS398, 0.03 for flurbiprofen and 1.9 for indomethacin.

In conclusion the present study has shown that cyclo-oxygenase inhibitors have a range of selectivities for cyclo-oxygenase 1 and cyclo-oxygenase 2 in intact human airway cells. The relative cyclo-oxygenase 2 selectivity of N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulphonamide and nimesulide may have implications for the treatment of asthma and other inflammatory pulmonary diseases.

Recent studies have suggested that cyclo-oxygenase (COX) products may be involved in the pathophysiology of several pulmonary diseases: COX products have been implicated in the inflammation found in asthmatic airways, with different COX products exerting differential effects in different situations in vivo [1–3]. Inhaled indomethacin and aspirin protect against indirect bronchoconstrictor challenges in asthma [4, 5] suggesting that release of constrictor prostaglandins such as prostaglandin (PG)D₂ and PGF₂α may contribute to the mechanisms of action of these stimuli. Under different circumstances COX products may have a protective role. PGE₂ has been shown to have a potential bronchoprotective role both in vitro [6, 7] and in vivo [8, 9]. Studies showing that oral indomethacin inhibits refractoriness to repeated bronchoconstriction challenge suggest that endogenous PGE₂ may be involved in this protective response [10, 11]. A small number of patients with asthma exhibit aspirin sensitivity, whereby oral aspirin and other COX inhibitors produce bronchoconstriction. This bronchoconstriction can be prevented by inhalation of PGE₂ [12, 13]. Collectively these studies implicate COX products in several aspects of asthma pathophysiology, but it is clear that the responses to COX inhibition depend on the inhibitor studied, the route of administration, the circumstances of use and the type of patients studied. Studies in cultured airway smooth muscle cells in vitro have suggested that COX products can also regulate adenylate cyclase function and the production of the chemokine interleukin (IL)-8 [14–16].

COX products have also been implicated in other pulmonary diseases: lung fibroblasts from patients with idiopathic pulmonary fibrosis have been shown to have a diminished capacity to synthesize PGE₂ [17]. Tracheal epithelial cells containing the DF508 cystic fibrosis (CF) mutation exhibit enhanced PG synthesis compared to wild type cells [18]. Further evidence that pro-inflammatory COX products may be involved in the pathophysiology of CF has been provided by a study showing that the COX inhibitor ibuprofen can slow the rate of pulmonary function decline in these patients [19]. In order to gain greater insight into the possible role of COX inhibitors in the modification of these and other pulmonary disease processes, further knowledge of the selectivity of COX inhibitors against COX isofoms in airway cells is required.

COX exists in two isoforms COX 1 and COX 2. COX 1 is constitutively expressed in many tissues [20], whereas COX 2 is an inducible form which has been shown to be induced in airway cells by stimuli present in inflammatory pulmonary diseases [21–23]. Airway cells from patients with asthma have been shown to express COX 2 in greater quantities than those from nonasthmatic subjects [24]. COX inhibitors are available with a range of selectivities...
for COX 1 and COX 2. The selectivity of these agents varies, however, between purified enzyme, broken cell and intact cell preparations [25]. A greater understanding of the effects of these agents in the lung requires study of their selectivity in whole cell preparations of airway cells.

COX isoforms are expressed in several airway cells. The authors and others have previously shown that COX 1 is the sole isoform expressed under resting conditions in human airway smooth muscle (HASM), and that COX 2 can be induced by cytokines, particularly IL-1β [22, 26, 27]. In contrast the epithelial cell line A549 expresses only COX 2 both under resting [28] and cytokine-stimulated conditions [21, 28]. Similar findings have been shown in primary cultures of airway epithelial cells [21, 28, 29].

Unstimulated HASM and A549 cells therefore represent good whole cell models for studies of COX 1 and COX 2 respectively. Therefore, the effects of a number of COX inhibitors were studied in these airway cells. The COX 1 selectivity of the drugs in unstimulated HASM cells that constitutively express COX 1 was examined along with the COX 2 selectivity in unstimulated A549 epithelial cells that constitutively express COX 2.

Materials and methods

Cell culture

Human airway smooth muscle cells. Primary cultures of adult HASM cells were prepared from explants of airway smooth muscle according to methods previously reported [22, 23, 30]. Briefly, human trachea was obtained from two post mortem individuals (one male aged 44 yrs and one female aged 52 yrs, with no evidence of airway disease) within 12 h of death. The trachealis muscle was then dissected free of epithelium and connective tissue under sterile conditions. (2 × 2 mm) explants of airway smooth muscle were then excised and ~10 explants placed in one small petri dish. The explants were incubated in 10% foetal calf serum (FCS)-Dulbecco’s modified Eagle’s medium (DMEM) in humidified 5% CO2/95% air at 37°C and the medium was changed every 3 days. Smooth muscle cells were usually seen ~7 days later. Once confluent, cells were trypsinized and plated into 175 cm2 tissue culture flasks, and growth-arrested for 24 h before experiments. Cells at passage 3 were used for all experiments. Small (2 × 2 mm) explants of airway epithelial cells were usually seen ~7 days later. Once confluent, cells were trypsinized and plated into 175 cm2 tissue culture flasks, and growth-arrested for 24 h before experiments. Cells at passage 3 were used for all experiments. Small (2 × 2 mm) explants of airway epithelial cells were usually seen ~7 days later. Once confluent, cells were trypsinized and plated into 175 cm2 tissue culture flasks, and growth-arrested for 24 h before experiments. Cells at passage 3 were used for all experiments.

A549 cells. A549 cells were purchased from the European Collection of Animal Cell Cultures (ECACC No: 86012804; Salisbury, Wiltshire, UK). Aliquots of cells frozen in 10% dimethyl sulphoxide (DMSO) - 90% FCS were thawed and suspended in culture medium comprising 90% DMEM - 10% FCS containing 2 mM l-glutamine, 100 μg·mL−1 penicillin G, 100 μg·mL−1 streptomycin and 2.5 μg·mL−1 amphotericin B. The DMSO was removed by centrifugation at 100 × g. Cells were resuspended in the above culture medium and plated in 12-well cultured plates at a seeding density of 1 × 105 cells·cm−2 and grown in 95% air/5% CO2 at 37°C. Culture media was replaced on alternate days until the cells were confluent. Cells were growth arrested for 24 h in media without FCS prior to all experiments.

Cyclo-oxygenase activity

Prior to each experiment, the culture medium was replaced with fresh culture medium without FCS. The COX inhibitor to be studied dissolved in DMSO (final concentration 1% volume/volume) or DMSO control was then added, and the cells were incubated at 37°C for 15 min. Exogenous arachidonic acid (AA; final concentration 5 μM) was then added and the cells were incubated for a further 30 min at 37°C. Media was removed and immediately frozen and stored at -20°C until assayed for PGE2. Six COX inhibitors were studied based on their selectivity in other systems: valeryl salicylate, a COX 1 selective inhibitor; nimesulide and N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulphonamide (NS 398), both COX 2 selective; and the nonselective COX inhibitors aspirin, indomethacin, and flurbiprofen.

Prostaglandin E2 assay

PGE2 concentration was determined by radioimmunoassay as previously described [31]. The authors have previously validated this assay [22, 23, 31] and have shown it to have a low cross-reactivity with other metabolites of AA [22, 23]. Each sample analysed for PGE2 was assayed in duplicate.

Protein assay

Cell protein was assayed in cell cultures used for Western blot analysis and relative COX activity. Following removal of the cell media for PGE2 estimation, extractable protein was removed from the cells by incubation with an extraction buffer (0.9% NaCl, 20 mM tris-HCl, pH 7.6, 0.1% Triton-X, 1 mM phenylmethylsulfonyl fluoride, 0.01% leupeptin) with gentle shaking. Extracted protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories Ltd, Hemel Hempstead, Hertfordshire, UK).

Western blot analysis of cyclo-oxygenase isoenzymes

Thirty micrograms of extracted protein was mixed 1:1 with sample buffer (20 mM tris-HCl, pH 6.8, 20% glycerol, 2% sodium dodecylsulphate (SDS), 5% 2-mercaptoethanol and 0.025% bromophenol blue) and boiled for 5 min prior to electrophoresis in 20 × 20 cm 7.5% SDS-polyacrylamide gel (45 mA, 5 h). Separated proteins were electrophroled in 150 V, 3 h) to pure nitricelulose membranes (Gelman Sciences, Northampton, Northamptonshire, UK) and the blot was blocked for 2 h at 4°C in blocking reagent (8% fat-free dried milk powder in phosphate-buffered saline (PBS) pH 7.4 with 0.3% Tween-20 (PBST)). The blot was then incubated with primary monoclonal anti-human COX 2 antibody (Cayman Chemical, Ann Arbor, MI, USA, 1:2,000 in blocking reagent) for 2 h at room temperature. The blot was subsequently washed with PBS-T and incubated with polyclonal anti-mouse immunoglobulin (Ig) G coupled with horseradish peroxidase.
The relative COX activity per unit of extractable protein of the two cell types was measured under growth arrested conditions. The results represent the mean±SEM of six experiments consisting of at least three replicates performed on separate days. PGE<sub>2</sub> production (5 μM AA, 30 min, 37°C) by HASM cells was 92±12 ng·mg<sup>-1</sup> protein, and 87±24 ng·mg<sup>-1</sup> protein by A549 cells.

**Cyclo-oxygenase inhibition**

COX activity was measured in both cell types following preincubation with selective and nonselective COX inhibitors at concentrations of 10<sup>-11</sup>–10<sup>-5</sup> M (fig. 2). COX activity median inhibitory concentration (IC<sub>50</sub>) values together with COX 2:COX 1 selectivity ratios for the COX inhibitors studied are shown in table 1. The COX activity of HASM cells could not be inhibited to 50% of baseline by 10<sup>-5</sup> M aspirin or 10<sup>-3</sup> M nimesulide, precluding accurate calculation of the IC<sub>50</sub> value. Aspirin 10<sup>-5</sup> M also failed to inhibit COX activity in A549 cells by 50%. Preincubation of both HASM and A549 cells with valeryl salicylate (10<sup>-11</sup>–10<sup>-8</sup> M) did not significantly inhibit COX activity (data not shown).

**Discussion**

COX inhibitors may have a clinical role in the treatment of a number of pulmonary inflammatory diseases. The current results show that HASM and A549 cells are good intact cell models for the study of the selectivity of COX inhibitors in pulmonary tissues in vitro. Under unstimulated conditions HASM and A549 cells exclusively express COX 1 and COX 2 protein, respectively. Western blotting was used to detect COX 1 and COX 2 protein. The authors feel confident that these results are an accurate reflection of the isoenzyme expression in these cells under unstimulated conditions and have previously shown that these antibodies are isoenzyme specific [22, 23]. Similar findings using Western blotting have previously been reported by the current authors and others [22, 23, 26–28]. Also previous studies using Northern blotting have confirmed the exclusive presence of COX 1 messenger ribonucleic acid (mRNA) in HASM cells [28, 29].

Direct comparisons between the absolute level of COX activity found in the current cells and previous work is difficult due to differences in methodology and the units of expression used. The only other study to compare COX activity in unstimulated HASM and A549 cells using similar methodology compared COX activity expressed per 10<sup>6</sup> cells [28], and found a similar level of COX activity in A549 cells but a >30-fold greater level of COX activity in A549 cells but a >30-fold greater level of COX activity in...
HASM cells. This may reflect differences present in the cell culture techniques used, or it may be due to the different site of origin of the HASM cells (cells used in the present study were primary cultures of tracheal smooth muscle cells, whereas those used by ASANO et al. [28] were a commercially available cell line of human bronchial smooth muscle cells).

In order to develop selective COX inhibitors for use in pulmonary disease it is desirable to have data on their selectivity in whole airway cells. The use of whole cells is especially important as marked differences in selectivity exist between purified enzyme and whole cell preparations [25]. Although many studies in airway cells have used COX inhibitors to probe the role of COX products in different experimental situations, the COX selectivity of these agents in airway cells has not been formally compared.

None of the COX inhibitors that were examined in the present study demonstrated COX 1 selectivity. Preincubation of both cell types with valeryl salicylate, a selective COX 1 inhibitor failed to produce significant COX inhibition. The authors believe this is most likely to be due to poor cellular uptake of this drug over the time course of the experiments. Although valeryl salicylate has been shown to inhibit COX 1 selectively in murine mast cells [32] extensive experimental data on this compound is lacking. Aspirin was the least potent of the other COX inhibitors studied. Aspirin 10^{-5} M did not produce a 50% inhibition of COX activity in either A549 or HASM cells (between 40% and 50% inhibition achieved for both cell types). This lack of potency compared with other COX inhibitors has been previously observed in other intact cell preparations [25, 33]. The level of COX 2 inhibition caused by aspirin in the present experiments was similar to that found in previous intact cell preparations, but the level of COX 1 inhibition was ~10-fold less than that previously reported [25, 33].

Fig. 2. – Inhibition of cyclo-oxygenase activity (5 μM arachidonic acid, 30 min, 37°C) assayed by prostaglandin (PGE2) production in human airway smooth muscle cells (▲) and pulmonary epithelial cell-line A549 cells (square) by: a) N-(2-cyclohexylosy-4-mitrophenyl)-methanesulphonamide (NS398); b) nimesulide; c) indomethacin; d) flurbiprofen; and e) aspirin. Data represents the mean±SEM of at least three separate experiments.
secretion of pro-inflammatory prostanoids, such as PGD2, to use COX 2 selective inhibitors to selectively reduce the inflammatory lung disease. It may therefore be possible to use COX 2 selective inhibitors to selectively reduce the secretion of pro-inflammatory prostanoids, such as PGD2 and PGE2α, without inhibiting the secretion of "housekeeping" prostanoids generated by COX 1.

Do the relative potencies of the COX inhibitors in airway cells help resolve some of the inconsistent or conflicting effects of these agents in asthma? The equal or greater affinity for the COX 1 isoenzyme by many COX inhibitors including aspirin and indomethacin may lead to preferential inhibition of "housekeeping" prostaglandin production (COX I). This might explain why aspirin may trigger asthma in a small minority of patients with "aspirin-sensitive" asthma. The finding that nimesulide (shown to be COX 2 specific in the current study) does not cause bronchoconstriction in aspirin-sensitive asthmatics [39] would be consistent with this. This group of patients are not representative of the majority of asthmatics however.

When given in high doses by inhalation to patients who do not have "aspirin sensitive" asthma, both aspirin and indomethacin have been shown to have protective effects against induced bronchoconstriction [4, 5, 40]. If this was a COX 1 effect, it would be unlikely to require such high concentrations given locally. This would suggest that it is mediated by COX 2 inhibition, and that the products of COX 2 are contributing to airway inflammation in the majority of asthmatics. Interestingly flurbiprofen demonstrated selectivity towards COX 2 in the present study and was the most potent of the three inhibitors in current use clinically: studies with flurbiprofen in asthma have shown that unlike aspirin and indomethacin it has a protective effect when given by the oral route [41].

In conclusion, it has been shown that cyclo-oxygenase inhibitors exhibit a spectrum of selectivity in intact pulmonary models of cyclooxygenase 1 and cyclo-oxygenase 2 expression. Drugs such as N-(4-cylohexylxoy-4-nitrophenyl)-methanesulphaonamide and nimesulide may prove useful in probing the role of cyclooxygenase 2 in asthma and other inflammatory lung diseases.

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References
