# COX-1, and not COX-2 activity, regulates airway function: relevance to aspirin-sensitive asthma

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ABSTRACT **Cyclooxygenase (COX) -1 and COX-2 are expressed in airway cells, where their activities influence functions such as airway hyperreactivity. Clinical data show that mixed COX-1/COX-2 inhibitors such as aspirin, but not COX-2 selective inhibitors such as rofecoxib, induce bronchoconstriction and asthma in sensitive individuals. This anomaly has not yet been explained. Here, we have used tissue from genetically modified mice lacking functional COX-1 (COX-1$^{-/-}$), as well as airway tissue from "aspirin-sensitive" and control patients to address this issue. Bronchi from wild-type mice contained predominantly COX-1 immunoreactivity and contracted *in vitro* in response to acetylcholine and U46619. Bronchi from COX-1$^{-/-}$ mice were hyperresponsive to bronchoconstrictors. Inhibitors of COX (naproxen, diclofenac, or ibuprofen) increased bronchoconstriction in tissue from wild-type but not from COX-1$^{-/-}$ mice. Cells cultured from aspirin-sensitive or control human donors contained similar levels of COX-1 and COX-2 immunoreactivity. COX activity in cells from aspirin-sensitive or tolerant patients was inhibited by aspirin, SC560, which blocks COX-1 selectively, but not by rofecoxib, which is a selective inhibitor of COX-2. These observations show that despite the presence of COX-2, COX-1 is functionally predominant in the airways and explains clinical observations relating to drug specificity in patients with aspirin-sensitive asthma.—Harrington, L. S., Lucas, R., McMaster, S. K., Moreno, L., Scadding, G., Warner, T. D., Mitchell, J. A. COX-1, and not COX-2 activity, regulates airway function: relevance to aspirin-sensitive asthma. *FASEB J.* 22, 4005–4010 (2008)**

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Cyclooxygenase (COX) -1 and COX-2 are coexpressed in the airways of healthy individuals, as well as in patients with asthma (1). Inhibition of COX enhances bronchoconstriction in mice (2). Furthermore, in a subgroup of patients with asthma, aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs) induce bronchoconstriction and acute asthma, resulting in the "aspirin-sensitive asthma" condition (3). It is thought that prostaglandin (PG) $E_2$, a product of COX, acts on prostaglandin E (EP) receptors on mast cells in the airways, providing a physiological brake against activation and histamine/leukotriene release (4). Thus, when aspirin and other NSAIDs are taken, COX is inhibited, the brake is removed, and airways are hypersensitive to antigens. Asthma is a chronic inflammatory disease in which COX-2 levels within the lung are elevated (5). Interestingly, despite COX-2 being present in the lung, COX-2 selective drugs, such as rofecoxib (Vioxx), do not induce asthma in these patients (6). This then presents the field with an anomaly—why do COX-2-selective drugs not induce asthma?

In the current study, we present novel and meaningful data that shed light on this important area. First, using tissue from COX-1$^{-/-}$ mice, we show that COX-1 is the functional isoform present in the airway with regard to bronchoconstriction. Second, using cells cultured from the airways of patients with aspirin-sensitive asthma, we show that despite detectable levels of COX-2, COX-1 is the functionally relevant form. Taken together, these observations provide a scientific rationale as to why COX-2 inhibitors are safe in asthma and illustrates a remarkably clear cut function of COX-1 in airway function.

## MATERIALS AND METHODS

### Mice and myograph studies

The COX-1$^{-/-}$ mice used in this study had an insertion into intron 11, resulting in transcription of an inactive "COX-1"

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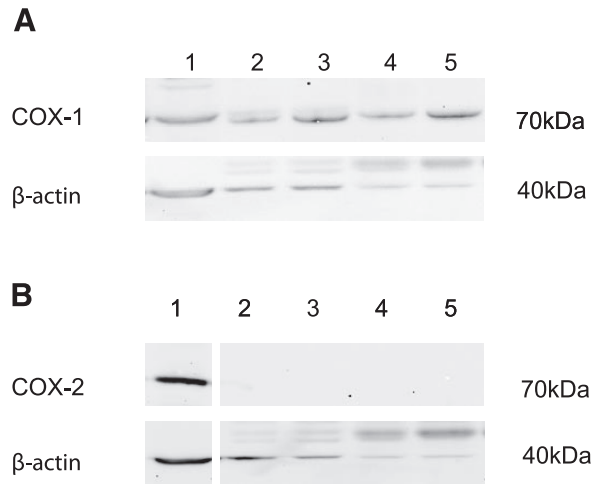


**Figure 1.** Western blot analysis of COX immunoreactivity in mouse bronchi. Tissue extracts of mouse bronchi contained immunoreactivity for COX-1 (*A*) with little or no detectable COX-2 (*B*). Positive (+ve) controls were extracts of MEG01 cells (22) for COX-1 and LPS-stimulated J774 cell extract (11) for COX-2. Blots were subjected to immune staining for β-actin as an indicator of protein loaded. Lane 1, positive control; lanes 2, 3, bronchi from wild-type mice; lanes 4, 5, bronchi from COX-$1^{-/-}$ mice. Similar results were obtained using tissue from a total of 3 or 4 mice.

protein (7). COX-$1^{-/-}$ mice were backcrossed for more than 7 generations onto a C57BL6 background. C57BL6 mice were used as wild-type controls. Male mice of 12–16 wk of age were used for this study. Mice were killed by cervical dislocation, before lungs were removed and dissected clear of connective tissue. Segments of main bronchi were loaded into wire myographs in warmed (37°C) and gassed (95%$O_2$:5%$CO_2$) physiological salt solution (PSS; NaCl, 119 mM; KCl, 4.7 mM; $CaCl_2$, 2.5 mM; $MgSO_4$, 1.17 mM; $NaHCO_3$, 25 mM; $KH_2PO_4$, 1.18 mM; EDTA, 0.027 mM; and glucose, 5.5 mM), as described previously (8). Isometric tension was initially calibrated to 0 mN. Optimal tensions for each bronchus were established by performing standard-length tension responses. In brief, bronchi were sequentially stretched by 0.25-mN increments (passive tension) and stimulated to contract actively (active tension) by the additions of a depolarizing concentration of potassium (124 mM). Optimal tension was taken at the point at which increased stretch ceased to increase active tension. Bronchi were then allowed to equilibrate for 20 min in PSS. Tissues were subjected to increasing concentrations of either acetylcholine or U46619, and tensions were recorded. In some experiments, NSAIDs or vehicle (dimethyl sulfoxide) were added for 30 min prior to the addition of acetylcholine or U46619.

### Human tissue

All subjects in the aspirin-sensitive asthma group had a history of aspirin-induced reaction, in that they developed a respiratory-type reaction following aspirin or NSAID ingestion. The reactions included bronchospasm, coughing, wheezing, and shortness of breath, as well as upper airway symptoms of rhinorrhoea and nasal obstruction. These patients had their aspirin sensitivity confirmed by an intranasal challenge with lysine-aspirin, as we have described previously (9). All patients (except for one) were receiving regular inhaled corticosteroids and $\beta_2$ adrenoreceptor stimulants, for use as required. Nasal polyp tissue was removed as part of outpatient surgery, as we have described previously (10). Tissue was dissected to remove the core inflammatory cell component, which is easily dissociated from surrounding stromal tissue and consists of inflammatory cells, including leukocytes and mast cells. Once the inflammatory core was removed the stromal airway tissue was chopped into small pieces and placed in to culture flasks containing DMEM supplemented with penicillin-streptomycin and 10% fetal calf serum. Cells began to grow out of the explants after 2–3 wk. Cells were fibroblast-like in appearance. Cells were plated into individual wells of either 96- or 6-well culture plates and grown in supplemented medium until confluent. For some experiments, cells were incubated with interleukin 1β (IL-1β; 1 ng/ml) for 24 h.

### Measurement of COX activity and protein expression

At the beginning of the experiment, cells were incubated with a range of concentrations of aspirin, SC560, or rofecoxib for 30 min before the addition of the calcium ionophore A23187 ($3\times10^{-5}$M) and incubation for a further 30 min. Medium was then removed, and COX activity over the incubation period was determined by measuring the level of $PGE_2$ within the medium using radioimmunoassay (11). COX protein expression was determined by Western blot analysis using the same protocol and antibodies as described previously for human (12) and mouse (13) tissues. β-Actin levels were estimated on blots previously probed for COX-1 or COX-2 using a monoclonal primary antibody raised to human β-actin peptide, detected by a secondary antibody to mouse immunoglobulin G (IgG) raised in goat (Abcam, Cambridge, UK).

## RESULTS

### COX-1 predominates in murine airway and mediates the prostaglandin brake to bronchoconstriction

Bronchi from wild-type mice contained predominantly COX-1 immunoreactivity (**Fig. 1**; 87.2±9.6% of COX-1 positive control; $n$=4). COX-1 immunoreactivity persisted in tissue from COX-$1^{-/-}$ mice (86.5±18.3% of COX-1 positive control; $n$=3), most likely explained by the presence in tissue from these mice of a nonfunctional protein product (7). No COX-2 immunoreactivity was detected in bronchi from either wild-type or

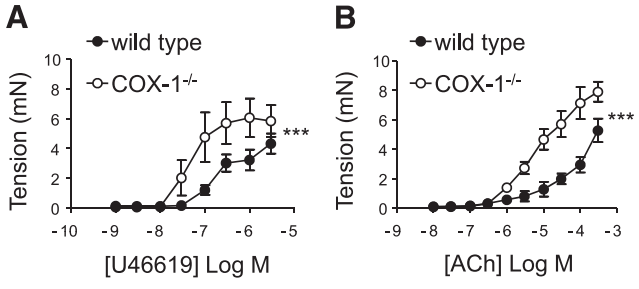


**Figure 2.** Comparison of bronchoconstriction induced by U46619 (*A*) or acetylcholine (ACh; *B*) in airways from wild-type (solid circles) or COX-$1^{-/-}$ (open circles) mice. Data are means ± SE; $n$ = 5 mice; 2-way ANOVA. ***$P < 0.001$.

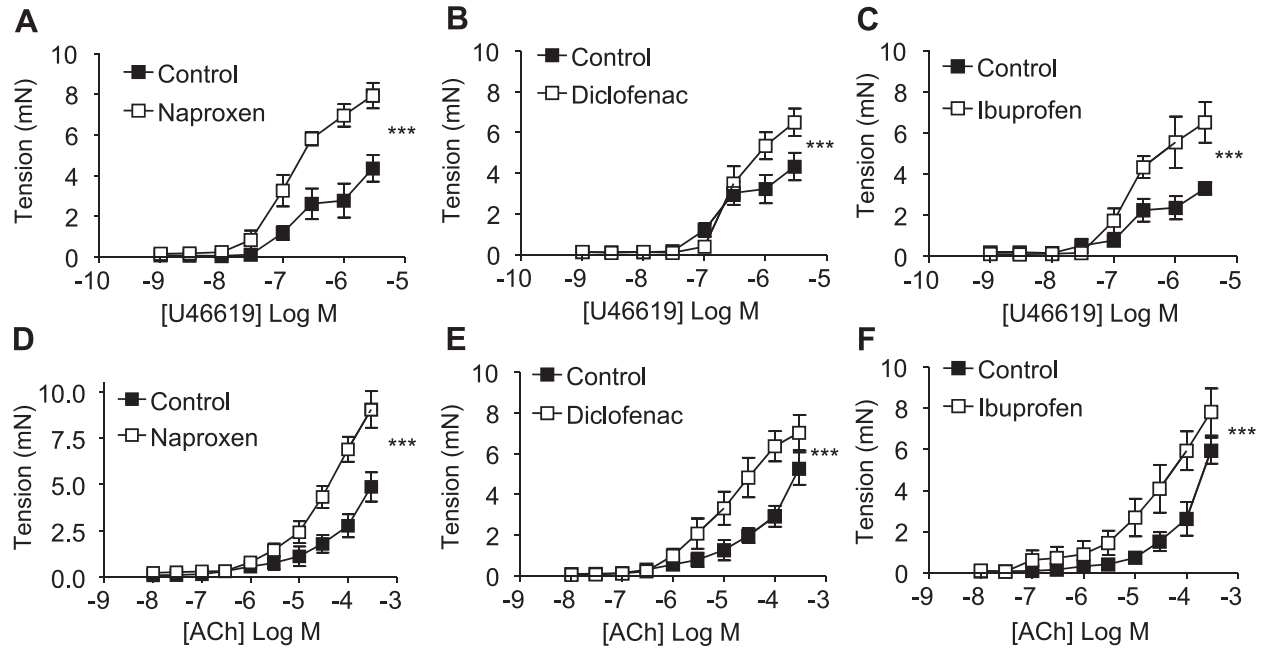


**Figure 3.** Effect of NSAIDs on bronchoconstriction induced by U46619 (*A–C*) or ACh (*D–F*) in airway tissue from wild type mice. The effects of the following NSAIDs were tested on constrictor responses to U46619 or ACh; naproxen ($10^{-4}$ M), diclofenac ($10^{-5}$ M), and ibuprofen ($10^{-5}$ M). Responses are means ± SE; $n = 4$ or 5 experiments; 2-way ANOVA. ***$P < 0.001$.

COX-1$^{-/-}$ mice (Fig. 1). Bronchi from wild-type or COX-1$^{-/-}$ mice contracted in response to increasing concentrations of U46619 ($10^{-9}$ to $3\times10^{-6}$ M; **Fig. 2*A***) or acetylcholine ($10^{-8}$ to $3\times10^{-4}$M; Fig. 2*B*). For both agonists, responses in bronchi from COX-1$^{-/-}$ mice were increased compared to those in bronchi taken from wild-type mice. Pretreatment of bronchi from wild-type animals with naproxen ($10^{-4}$ M), diclofenac

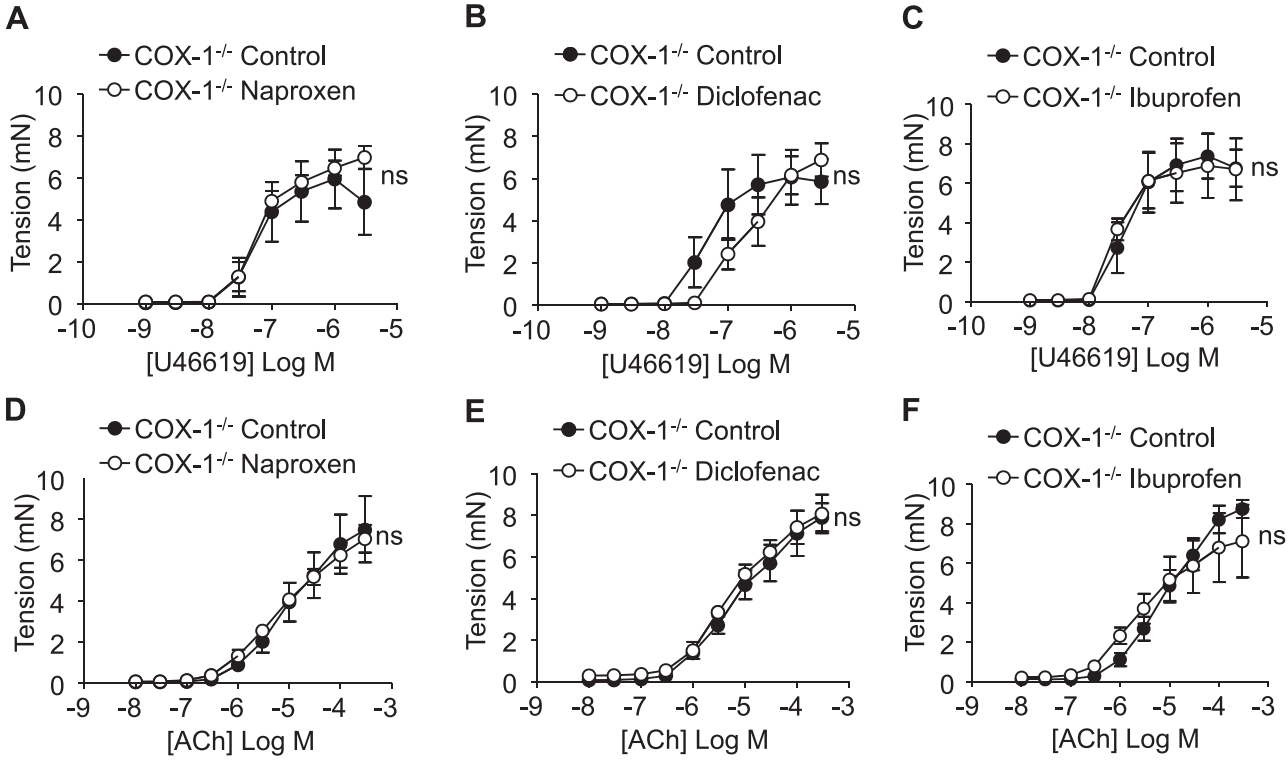


**Figure 4.** Effect of NSAIDs on bronchoconstrictions induced by U46619 (*A–C*) or ACh (*D–F*) in airway tissue from COX-1$^{-/-}$ mice. The effects of the following NSAIDs were tested on constrictor responses to U46619 or ACh of naproxen ($10^{-5}$ M), diclofenac ($10^{-5}$ M), and ibuprofen ($10^{-4}$ M). Responses are means ± SE for $n = 4$ or 5 experiments; 2-way ANOVA. ns, nonsignficant difference ($P>0.05$).

($10^{-5}$ M), or ibuprofen ($10^{-5}$ M) increased bronchoconstrictor responses to both U46619 (**Fig. 3*A–C***) and acetylcholine (**Fig. 4*A–C***). In contrast, pretreatment of bronchi from COX-$1^{-/-}$ mice with ibuprofen or naproxen had no significant effect on contractions induced by either U46619 (Fig. 3*D–F*) or acetylcholine (Fig. 4*D–F*).

### Relation between COX-1 and COX-2 in nasal airway tissue from aspirin-tolerant and aspirin-sensitive patients

Similar to observations made with murine airway tissues, cells cultured from nasal polyps obtained from aspirin-sensitive or aspirin-tolerant patients contained predominantly COX-1 protein. However, although in limited amounts, COX-2 immunoreactivity was detected in cells from both patient groups (**Fig. 5*A***). In contrast to some other types of human cells [*e.g.*, A549 (12, 14), endothelial (15), vascular smooth muscle (16)], the cytokine IL-1β did not induce increased expression of COX-2 in nasal polyp cells (Fig. 5*A*). Cells cultured from control and aspirin-sensitive individuals released detectable levels of $PGE_2$ over 24 h in culture (Fig. 5*B*). There was no significant difference in absolute levels of $PGE_2$ released by cells from the two patient groups cultured under control conditions (Fig. 5*B*). Despite having little or no effect on protein expression, treatment of cells with IL-1β for 24 h increased the release of $PGE_2$. $PGE_2$ release after IL-1β was significantly higher from cells cultured from aspirin-sensitive than from aspirin-tolerant individuals (Fig. 5*B*).

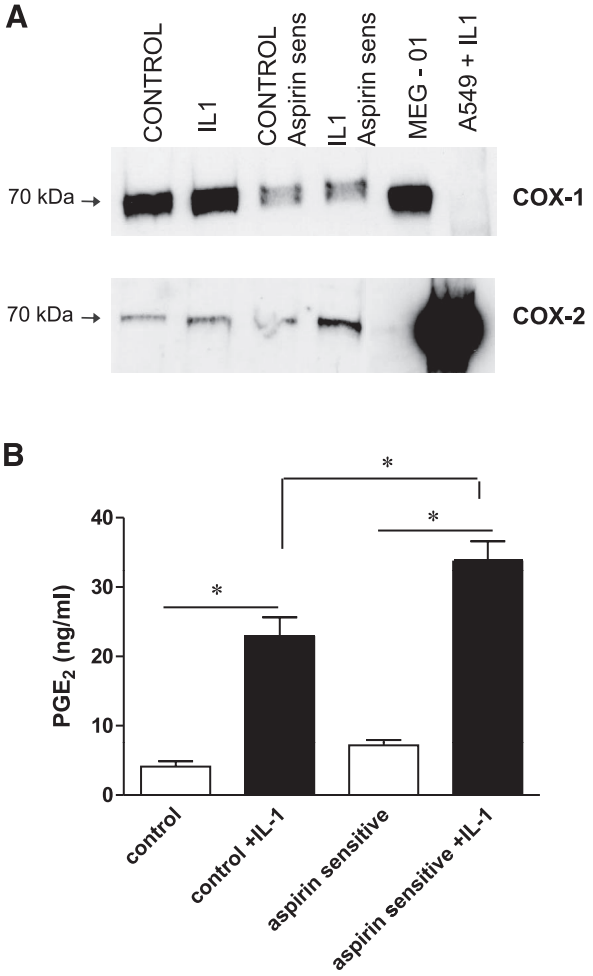


**Figure 5.** Relative levels of COX-1 and COX-2 expressed in human cells derived from control (no aspirin sensitivity) and aspirin-sensitive donors and cultured under control conditions or in the presence of IL-1β for 24 h. COX-1 and COX-2 immunoreactivity was detected in extracts from control cells and those treated with IL-1β (A). Positive controls used for this blot were MEG-01 cytosol, which contains only COX-1 (22), and A549 cells treated with IL-1β, which express only COX-2 (23). COX activity in cells measured by release of $PGE_2$ into the medium after incubation with A23187 ($10^{-5}$ M) for 30 min (*B*). Data are means ± SE; $n = 15$ experiments (cells from at least 3 different patients; experiments performed in triplicate on at least 5 experimental days); 1-way ANOVA with *post hoc* test. $^*P < 0.05$.

### Pharmacological characterization of COX isoforms in nasal airway tissue from aspirin-tolerant and aspirin-sensitive patients

COX activity in human cells cultured from aspirin-sensitive and aspirin-tolerant individuals was inhibited by aspirin (**Fig. 6*A***) and the COX-1 selective inhibitor SC560 (Fig. 6*B*). The potency and efficacy of aspirin and SC560 was identical in cells cultured from the two patient groups. In contrast to results with aspirin or SC560, the COX-2-selective drug rofecoxib was inactive as an inhibitor of $PGE_2$ production in cells derived from both aspirin-sensitive and aspirin-tolerant individuals (Fig. 6*C*).

## DISCUSSION

Asthma is a chronic inflammatory disease characterized by inflammation of the airways and enhanced bronchoconstrictor responses. COX-1 and COX-2 are expressed in the airways of patients with asthma. However, unlike some other chronic inflammatory diseases, such as arthritis, inhibition of COX with NSAIDs does not provide any anti-inflammatory or analgesic relief in asthma. In fact, for a subset of patients with asthma, ingestion of NSAIDs induces asthma (aspirin-sensitive responders). Paradoxically, while NSAIDs induce asthma in sensitive individuals, selective COX-2 inhibitors appear to be well tolerated (6).

In the current study, we confirm what is already known about the effects of NSAIDs on airway responses in tissue from wild-type mice. Pretreatment of bronchi with NSAIDs consistently increased the bronchoconstrictor effects of the thromboxane mimetic U46619 or acetylcholine. NSAIDs increase bronchoconstrictor responses by two potential mechanisms, both of which involve inhibition of prostaglandin synthesis. First, prostaglandins produced by COX (including $PGE_2$) functionally antagonize airway contraction and secondly tonically suppress the synthesis and release of

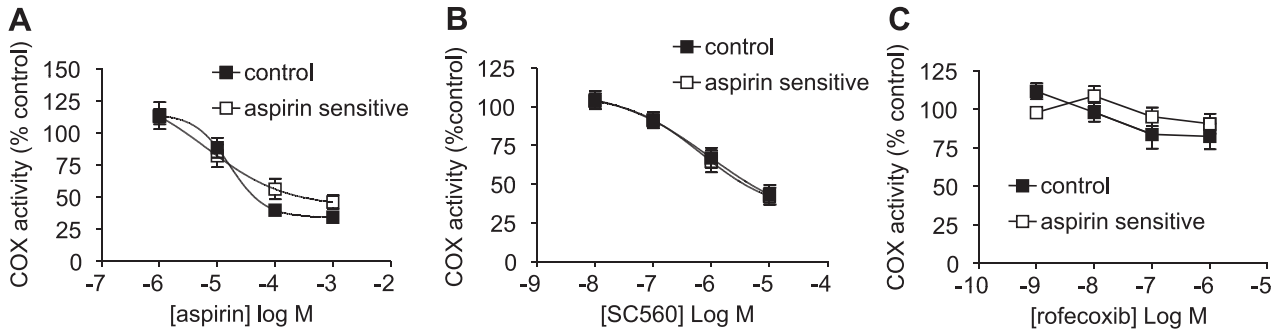


**Figure 6.** Effect of aspirin (*A*), SC560 (*B*), or rofecoxib (*C*) on COX activity in cells from control (solid squares) or aspirin-sensitive patients (open squares). Data are means ± SE; $n = 15$ experiments (cells from at least 3 different patients; experiments performed in triplicate on at least 5 experimental days).

bronchoconstrictor leukotrienes (17). NSAIDs inhibit both COX-1 and COX-2; it is, therefore, not possible to establish which isoform of COX predominates by the use of NSAIDs alone. Here, we found that airway tissue from laboratory mice contained predominantly COX-1, with no detectable COX-2 expression. In line with this, we found that the ability of NSAIDs to increase bronchoconstrictor responses in mouse airways was completely lost in tissue from COX-$1^{-/-}$ mice. Although this observation may, on the face of it, seem predictable from our studies on COX expression, it was essential to check. Low levels of COX-2 may have been strategically compartmentalized—and therefore difficult to detect by Western blot analysis. Alternatively, NSAIDs may have been affecting airway responses by non-COX-dependent mechanisms known to be active in some tissues (18). However, our data clearly and definitively show that in mouse airways, COX-1, and not COX-2, is the target for NSAID action. It is conceivable that disruption of the COX-1 gene could influence COX-2 expression as a compensatory mechanism. However, as in tissue from wild-type animals, we found no detectable COX-2 in airway samples from COX-$1^{-/-}$ mice.

In human airway tissue cultured from nasal polyps, we detected both COX-1 and COX-2. As was the case for mouse tissue, COX-1 predominated. This was true for tissue from non-aspirin-sensitive donors, as well as from patients with aspirin-sensitive asthma. These observations are consistent with others in the literature showing that both isoforms of COX are present in the airways of patients with aspirin-sensitive asthma (5). In our study, we went further, and investigated the functional capacity of COX in human airway cells and the COX isoform expressed. As expected, nasal polyp cells released detectable levels of $PGE_2$ when stimulated with the calcium ionophore A23187. Interestingly, although activation of cells with IL-1β did not induce COX-2 in these cells, it did increase the production of $PGE_2$ by the cells. This was true for cells cultured from both aspirin-sensitive and control subjects. Interestingly, after incubation with IL-1β, cells derived from patients with aspirin-sensitive asthma release higher levels of $PGE_2$ than cells cultured from control donors. Clearly, this was not mediated by increased expression of COX and most likely, therefore, was due to increased expression of phospholipase $A_2$ (19), the enzyme responsible for liberation of the substrate arachidonic acid.

In a previous study from our group using blood donated by these patients, we found that COX-1 or COX-2 was similarly inhibited by NSAIDs, including aspirin, irrespective of aspirin sensitivity (9). In the current study, we found that airway cells from aspirin-sensitive and aspirin-tolerant donors were inhibited similarly by aspirin or by SC560. Aspirin inhibits both COX-1 and COX-2 (11, 20), whereas SC560 is a highly selective inhibitor of COX-1 (21). Again, as with aspirin, the potency and efficacy of SC560 were identical in cells from aspirin-sensitive and control donors. Finally, we found that, despite the presence of detectable levels of COX-2, the COX-2-selective inhibitor rofecoxib, was inactive as an inhibitor of COX in cells derived from either aspirin-sensitive patients or control donors.

In summary, our findings using genetically modified animals and tissue from aspirin-sensitive individuals show that COX-1 is the dominant isoform in the airways, particularly with regard to mechanical function. Notably, these data explain why aspirin and other NSAIDs that inhibit COX-1, but not drugs that selectively inhibit COX-2, can be tolerated by aspirin-sensitive patients with asthma. FJ

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