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Original Article

A Cyclooxygenase-2 Selective Inhibitor Worsens Respiratory Function and Enhances Mast Cell Activity in Ovalbumin-Sensitized Mice

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ABSTRACT

Background: Cyclooxygenase (COX)-2 activity has been said to have a protective effect in asthmatic patients as a result of prostaglandin E_2 production. In order to elucidate the mechanisms involved, we evaluated the impact of selective inhibition of COX-2 with rofecoxib during ovalbumin (OVA) challenge, assessing mast cell activity and airway response in a murine model of asthma.

Material and methods: Mice were sensitized to OVA (10 μg injected intraperitoneally) and further challenged with 0.5% intranasal OVA. Half the sensitized animals were treated orally with rofecoxib (15 mg/kg/d during the challenge phase). Lung function was measured by whole body plethysmography before and after exposure to OVA. The severity of airway inflammation was evaluated by means of a scoring system. Finally, the serum level of mouse mast cell protease (mMCP)-1 was determined as an indicator of mucosal mast cell activity.

Results: Sensitized mice treated with rofecoxib exhibited 2.4-fold greater airway hyperresponsiveness than did vehicle-treated mice at a methacholine concentration of 100 mg/mL. A clear trend toward worsening airway inflammation in the presence of rofecoxib was observed, although the difference between rofecoxib-treated and vehicle-treated animals was not significant. These changes were accompanied by a significant increase in mucosal mast cell activity.

Conclusions: Selective pharmacological inhibition of COX-2 during the challenge phase worsens airway function in the OVA-induced murine model of acute asthma. We suggest that this effect might be at least partially explained by the increase in airway mast cell activity.

Un inhibidor selectivo de la ciclooxigenasa-2 empeora la función respiratoria y fomenta la actividad de los mastocitos en ratones sensibilizados a la ovalbúmina

RESUMEN

Introducción y objetivo: Se ha señalado que la ciclooxigenasa-2 (COX-2) ejerce una función protectora en pacientes con asma mediante la producción de la prostaglandina E_2. Con el objetivo de reproducir dicho efecto en un modelo experimental y dilucidar los mecanismos implicados, hemos evaluado, en un modelo de asma alérgica en el ratón, el efecto de la inhibición de la COX-2 en la respuesta de las vías aéreas expuestas a ovalbúmina y en la actividad de los mastocitos.

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Material y métodos: Se sensibilizaron ratones a la ovalbúmina (10 μg, vía intraperitoneal) y se reexpusieron a ovalbúmina al 0,5% por vía intranasal. La mitad de los animales sensibilizados recibió tratamiento con rofecoxib (15 mg/kg al día, por vía oral, durante la fase de reexposición). Se evaluó la función pulmonar mediante pletismografía corporal antes y después de la reexposición a ovalbúmina, y se estableció el grado de inflamación broncovascular. También se midió la concentración sérica de la proteasa-1 de los mastocitos de ratón.

Resultados: Los ratones sensibilizados y tratados con rofecoxib mostraron una hiperreactividad bronquial 2,4 veces mayor que los del grupo control a una concentración de 100 mg/ml de metacolina. Asimismo, se apreció una clara tendencia hacia el empeoramiento del proceso inflamatorio en presencia de rofecoxib, aunque sin significación estadística. Estos cambios se acompañaron de un aumento significativo de la actividad de los mastocitos de la mucosa.

Conclusiones: La inhibición farmacológica de la COX-2 durante la reexposición a ovalbúmina agrava la función pulmonar, un fenómeno que consideramos se debe, al menos en parte, al incremento de la actividad de los mastocitos de las vías aéreas.

Introduction

Prostaglandins are considered potent proinflammatory mediators and prostaglandin E_2 (PGE_2) is known to induce edema and hyperalgesia during an inflammatory insult.[1,2] Prostaglandins are generated via the metabolism of arachidonic acid by cyclooxygenase (COX), which has at least 2 isoforms: COX-1 and COX-2. Although COX-1 is constitutively expressed, COX-2 is induced by proinflammatory stimuli and is thus crucial to the inflammatory response. Nonsteroidal anti-inflammatory drugs (NSAIDs) that selectively inhibit COX-2 have been proposed as a treatment for managing inflammatory diseases,[3] specifically in NSAID-intolerant patients.[4,5]

In asthma, COX-2 activity has been implicated in the development of airway hyperresponsiveness through its ability to produce the airway smooth muscle bronchoconstrictor prostaglandin D_2.[6] However, evidence has emerged that prostaglandins such as PGE_2, and therefore possibly COX-2, can exert protective effects in asthma. For instance, inhaled PGE_2 attenuates or even completely abrogates the bronchospasm precipitated by allergens, exercise, and NSAIDs in asthmatic patients.[7-9] Accordingly, the refractory period that usually follows an initial episode of exercise-induced bronchospasm appears to be shortened by the administration of a COX inhibitor.[10,11] Consistent with these in vivo observations, airway cells from aspirin-induced asthma patients have been widely shown to have a reduced ability to produce either COX-2 or PGE_2 in vitro.[12-17] Furthermore, a stronger airway response upon exposure to the allergen has been observed in animals with COX-1 and/or COX-2 pharmacologic or genetic blockade in comparison with control animals.[18-22]

Although these observations support a protective role for COX-2 products against inflammation and bronchospasm, the mechanisms involved are far from well understood. Airway mast cells, which are known to be involved in the development of asthma,[23] bear PGE_2 receptors on their surface, where some have been proven to reduce mast cell activity in vitro upon activation.[24,25] Since mast cells produce a wide array of proinflammatory and bronchotropic mediators,[23] the possible protective effect of COX-2 on the asthmatic response in vivo may be mediated by a restraining effect of PGE_2 on these cells. Accordingly, the observed worsening of airway inflammation in COX-deficient murine models[19] could be explained by enhanced mast cell activity.

In order to further assess the role of COX-2 in the sensitized airway response and the relevance of mast cells to it, we inhibited COX-2 by means of the selective drug rofecoxib in ovalbumin (OVA)-sensitized mice. The drug was administered during the OVA challenge phase only and its impact on airway function, inflammation, and mucosal mast cell activity was assessed.

Materials and Methods

OVA-Sensitized Mouse Model

The animal studies were performed under the approval of the ethics committee of Universitat Autònoma de Barcelona. Adult female Balb/c mice (6-8 weeks old) were exposed to OVA (Sigma, Madrid, Spain) using a modification of the well-established protocol of Kobayashi et al.[26] Briefly, initial sensitization to OVA was achieved through 2 intraperitoneal injections of 10 μg of OVA adsorbed to 1 mg of aluminum hydroxide (Pierce, Rockford, Illinois, USA) on days 0 (first day of the study) and 5, followed by further multiple challenges via aerosol nebulization for 20 minutes, with a 5 mg/mL (0.5%) OVA solution on days 12, 15, 18, 21, and 22.

Selective COX-2 Blockade

Mice were distributed in 2 experimental groups. One consisted of OVA-sensitized mice which were treated only with vehicle (untreated, n=8). The other consisted of OVA-sensitized mice treated with the selective COX-2 inhibitor rofecoxib (n=7). Animals from these 2 groups were handled identically except for the application of a treatment, which was either rofecoxib (Vioxx, MSD, Madrid, Spain) or vehicle (sorbitol 15 g in 5 mL of saline solution). Rofecoxib was given by oral gavage in order to ensure administration of the full dose of 15 mg/kg in all the treated mice. Starting 1 day before the first challenge exposure to OVA (ie, day 11), the drug was administered for 14 consecutive days, after which time the animals were sacrificed. The treated mice had therefore received rofecoxib throughout the allergen challenge process but not during the initial sensitization phase. The mice were observed daily for potential adverse effects to the drug as per a safety protocol that included assessment of the appearance of the animal's coat, body position, presence of secretions, behavior, and abnormal breathing.

Assessment of Airway Function

Airway function was assessed in mice in both groups using noninvasive whole body plethysmography (WBP, Buxco, Winchester, UK). This allowed airway reactivity to methacholine to be evaluated twice within the same animal, once before and once after exposure to OVA. Airway function measured before sensitization (ie, on day 2 of the study) was taken as the baseline reference for bronchial responsiveness. Airway reactivity in the OVA-sensitized mice was also assessed on day 23 of the study 24 hours after the last OVA challenge. Briefly, conscious mice from both groups were placed in the controlled airflow plethysmography chambers and exposed to nebulized phosphate buffered saline (PBS) followed by subsequent increasing doses of

nebulized methacholine (3, 10, 30, and 100 mg/mL). The reaction of the airways to methacholine was averaged from readings taken over a 5-minute recording period and expressed as the enhanced pause value (Penh), a dimensionless indicator of airway hyperreactivity.[27,28] The methacholine concentration resulting in a 2-fold increase of the baseline Penh effect (EC_{20}) was also calculated and compared between groups.

Assessment of Airway Inflammation

A histologic assessment of the lungs was performed in OVA-sensitized and nonsensitized mice to evaluate underlying inflammation. Briefly, on day 24, OVA-exposed mice from both the rofecoxib-treated and vehicle-treated groups were sacrificed together with 3 nonsensitized animals used as a control for the histology assessment. The left lung was excised, the airways perfused to remove any obstruction, and the tissue immersed in 10% formaldehyde for histologic processing. The tissue was embedded in paraffin and 6 sections (4 μm) were obtained from 3 different areas of each animal's lung. The sections were then stained with hematoxylin and eosin to assess inflammation. A scoring system that took into account both the number of focal accumulations of inflammatory cells around bronchovascular structures, and their size was used to assess inflammation. Focal accumulations, defined as sites where inflammatory cells clustered around a bronchus and/or a vessel, were counted by 2 experienced researchers who were blinded to group assignment. Briefly, 4 bronchovascular structures per section were selected randomly, inflammatory accumulations per structure were counted, and a score was assigned (a score of 0 for 1 accumulation, 1 for 2-3 accumulations, 3 for 4-5 accumulations, and 5 for 6 or more accumulations). To assess the size of the focal accumulations, cell layers within the largest accumulation around each structure were counted and a similar scoring system was applied (a score of 1 for 1-4 layers, 3 for 5-9 layers, 5 for 10-16 layers, and 7 for 17 or more layers). The severity of inflammation was expressed as the sum of the 2 scores.

Assessment of Mast Cell Activity

Mucosal mast cells are the predominant cell population in the airways.[29] Their activity can be evaluated by determining the concentration of the mucosal mast cell-specific mediator, mouse mast cell protease-1 (mMCP-1). Briefly, on day 24, sensitized mice from both groups were exsanguinated, and sera were frozen at –20°C until analysis. The serum mMCP-1 concentration was measured using a specific enzyme-linked immunoassay kit according to the manufacturer's instructions (MS-RM3, Moredun Scientific Ltd, Penicuik, Scotland).

Calculations and Statistics

Airway reactivity to methacholine was expressed as a fold increase of the Penh value following exposure to methacholine in comparison with PBS. Two statistical comparisons followed: *a)* the Penh fold increase before sensitization to OVA was compared to the increase afterwards within the same animals using a paired *t* test, and *b)* the Penh fold increase of the rofecoxib-treated sensitized mice was compared to that of the untreated sensitized mice using an unpaired *t* test. Finally, the severity of inflammation and the mMCP-1 concentrations were also compared between groups with an unpaired *t* test. In all cases the established level of statistical significance was α= .05.

Results

No clinical, behavioral, postural, or phenotypic differences between treated and untreated mice were observed after oral rofecoxib administration.
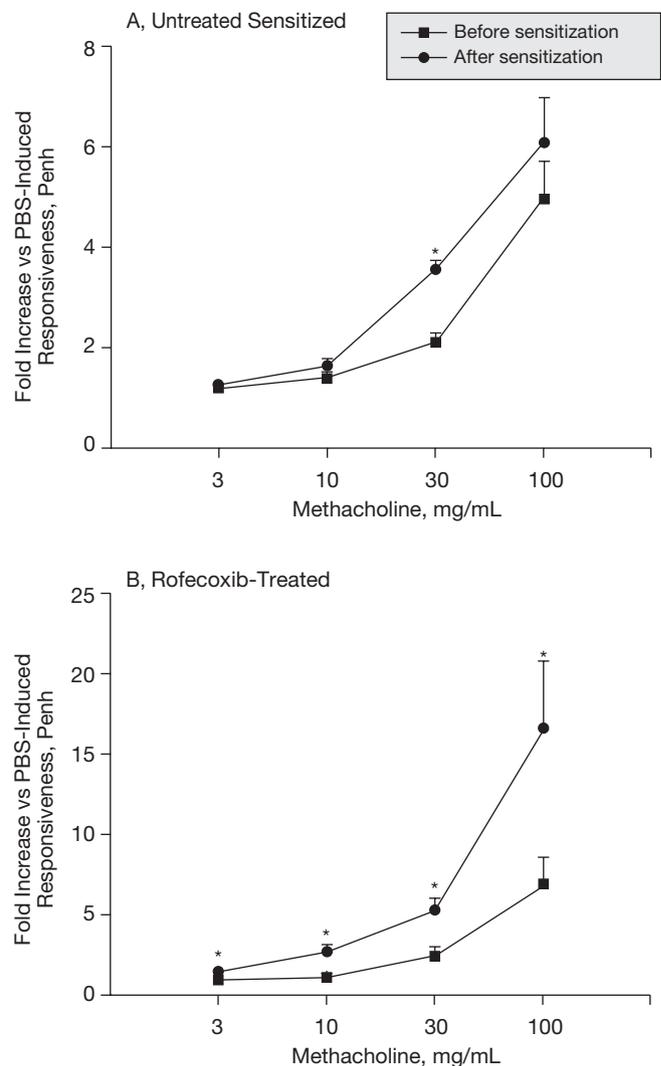

Figure 1. Bronchial response to increasing methacholine concentrations in untreated (A) and rofecoxib-treated (B) mice before (squares) and after (circles) airway sensitization with ovalbumin (OVA). The reaction to methacholine prior to OVA sensitization was similar in both groups (squares in both graphs). The airway hyperresponsiveness of the untreated sensitized mice reached a mean (SEM) Penh fold increase of 6.94 (1.80) at a methacholine dose of 100 mg/mL; this level was significantly below the Penh fold increase of 16.86 (4.04) in the rofecoxib-treated sensitized mice at the same methacholine concentration. Note that the scales on the *y* axes in graph A and B differ because of the different order of magnitude of airway responses to methacholine in the 2 groups.
Data represented are means, and error bars correspond to the SEM; significance was set at *P*<.05* (paired *t* test comparing Penh before and after OVA sensitization).

Airway Function

The airway response to methacholine of both rofecoxib-treated and untreated mice, both before and after OVA sensitization, is shown in Figure 1. The bronchial response to increasing methacholine concentrations before the sensitization to OVA was similar in both groups. As indicated in Figure 1, the Penh of both rofecoxib-treated and untreated mice increased on exposure to OVA. However, the airway hyperresponsiveness, as shown by the Penh fold increase across the methacholine dose-response curve, was significantly greater in the rofecoxib-treated OVA-sensitized mice. These data were confirmed by EC_{20} comparisons of the difference in the mean (SEM) EC_{200} in untreated (7.62 [0.5] mg/mL) and rofecoxib-treated (3-34 [0.62] mg/mL) OVA-sensitized mice; this difference was statistically significant (*P*=.0005).
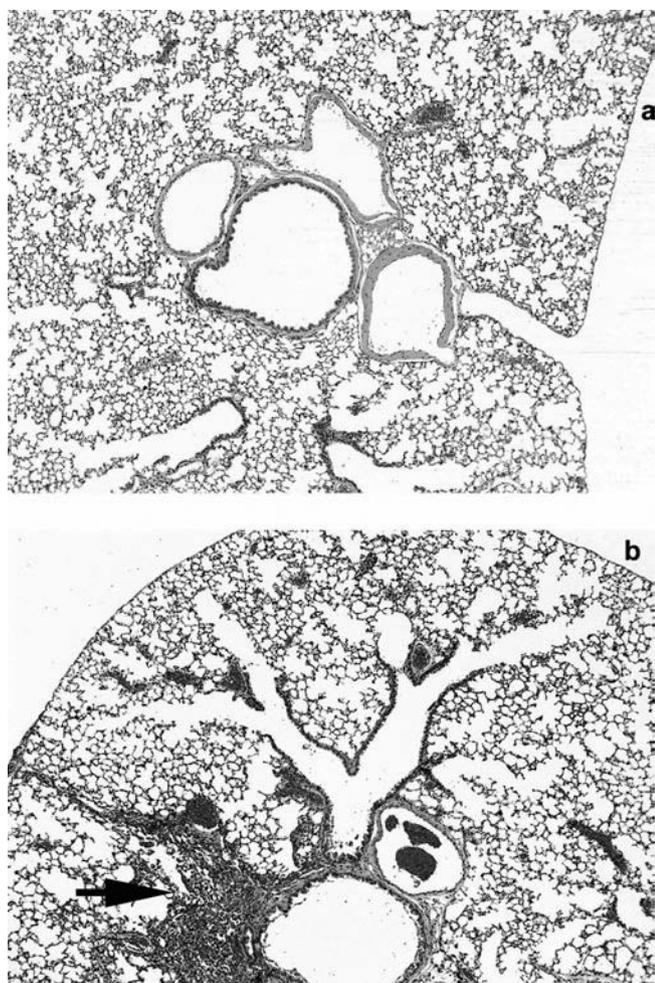
Figure 2. Microphotographs of the lower airways of nonsensitized (A) and untreated sensitized (B) mice. The nonsensitized animals showed no sign of inflammation whatsoever, while the sensitized animals developed focal accumulations of inflammatory cells around bronchovascular structures (arrow). The infiltrate largely consisted of mononuclear cells with a high number of eosinophils. The images are representative of observations from the studied groups.

Airway Inflammation

The scoring was reproducible with high interobserver agreement (r^2=0.99, P<.001). Both rofecoxib-treated and untreated OVA-sensitized mice developed an inflammatory response, whereas the nonsensitized animals used as controls for the histologic assessment showed no signs of inflammation (Figure 2). Eosinophils and mononuclear cells were predominant in the perivascular and peribronchial areas of the inflamed lungs. Inflammation normally appeared as focal inflammatory cell accumulations around the bronchovascular structures, but disseminated cells were also occasionally observed. The graph in Figure 3 depicts the level of inflammation in rofecoxib-treated and untreated OVA-sensitized mice, quantified by means of the scoring system described earlier. There was a trend (P=.09) towards higher scores in the rofecoxib-treated animals; however, the difference between the treated and untreated mice was not significant. An analogous result was obtained for eosinophil counts per square millimeter (area of the histologic section): a mean of 6.22 (2.0) cells/mm^2 were seen in vehicle-treated animals and 10.69 (3.47) cells/mm^2 in rofecoxib-treated ones (P=.2). Finally, a significant correlation was observed between the airway response to 100 mg/mL of methacholine and the level of inflammation in the sensitized mice (Figure 4) (r^2=0.38, P=.019).
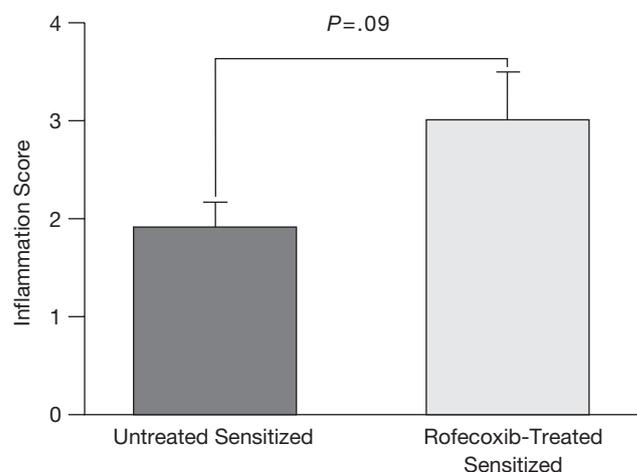

Figure 3. Level of inflammation in the lungs of sensitized mice treated with the selective COX-2 inhibitor rofecoxib and sensitized control animals treated with vehicle in saline solution. The inflammation score was higher in the rofecoxib-treated mice, but the difference did not achieve statistical significance (P=.09). Data represented are means and error bars correspond to SEM.

Mast Cell Activity

As shown in Figure 5 the mMCP-1 serum concentration was more than 2-fold higher in the rofecoxib-treated OVA-sensitized animals than in untreated ones (9.4 [2.3] ng/mL vs 4.3 [0.8] ng/mL, respectively).

Discussion

We have shown that selective pharmacological blockade of COX-2 activity with rofecoxib during the OVA challenge phase in a murine model of acute asthma increases the airway response to methacholine, and that such increase is accompanied by enhanced activity of the mucosal mast cells. Our results also suggest that the underlying airway inflammation worsens in sensitized mice treated with the selective COX-2 inhibitor.

The use of a non-invasive technique, whole body plethysmography, to assess airway function allowed us to measure bronchial reactivity in the same mice before and after airway sensitization. The mice therefore served as their own controls within each experimental condition. Using this approach we found that the COX-2 inhibitor rofecoxib caused worsening of airway hyperreactivity in the sensitized mice on 2 levels. Firstly, it induced a more pronounced response to methacholine. Secondly, it favored the development of a response at a lower methacholine concentration, as indicated by the point at which the effect increased 200%, which revealed enhanced airway reactivity in the presence of rofecoxib. Interestingly, mast cell activity more than doubled under rofecoxib as determined by the sustained increase in the serum concentration of mMCP-1 even 48 hours after the last exposure to OVA. Although a causal relation cannot be firmly established under our experimental approach, it appears likely that there is a direct link between the observed increase in mast cell activity and enhanced bronchial reactivity.[26] Additional experiments would be required to elucidate the relationship between rofecoxib and mast cells; nonetheless, it is possible to tentatively propose that the mechanism underlying the observed increase involves the expression of inhibitory PGE_2 receptors by airway mast cells.[24,25] Thus, a potential decrease in PGE_2 production caused by rofecoxib may abolish the inhibitory effect that this prostaglandin might be exerting on those cells, upregulating mast cell activity. Under this hypothesis, even though COX-2 selective inhibitors are generally
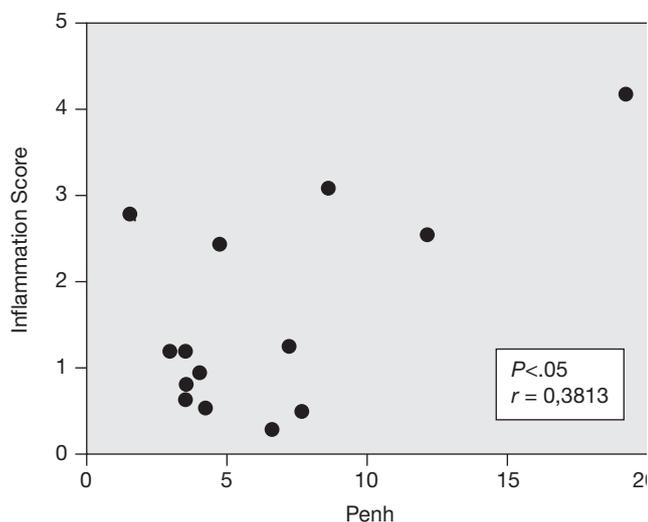
Figure 4. Significant correlation between airway reactivity (Penh) to a methacholine concentration of 100 mg/mL and the level of inflammation (score) ($P<.05$ for a coefficient of determination (r^2) of 0.3813, ie 38% of the Penh variability can be explained by the variation of inflammation).
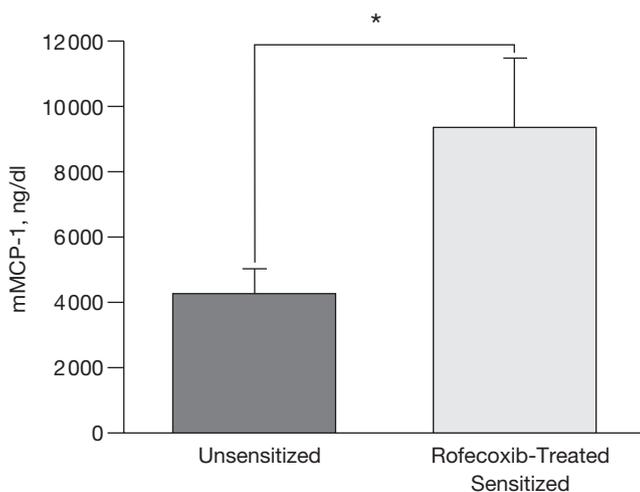

Figure 5. Serum mouse mast cell protease-1 (mMCP-1) concentration in rofecoxib-treated and untreated sensitized mice measured by enzyme-linked immunoassay 48 hours after the last exposure to ovalbumin ($P<.05^*$). Data represented are means, and error bars correspond to the SEM.

regarded as safe in aspirin-induced asthma patients,[4,5] the relevance of mast cells in mediating, at least partly, NSAID-induced adverse effects through a similar mechanism should be further analyzed. Finally, it is noteworthy that although an additional experimental group of rofecoxib-treated but nonsensitized mice would have helped rule out a possible direct NSAID-driven effect on the airway reactivity in these mice, we believe this possibility to be unlikely since there are no reports of this or other COX-2 inhibitors having effects on either regular airway function or increased mast cell activity in mice or humans. Although other authors have found that selective COX inhibitors accentuate airway hyperreactivity to intravenous methacholine,[20,21] mast cell modulation was never suggested to be a contributing factor. We propose a mast-cell-dependent mechanism to explain the worsening of airway hyperreactivity under pharmacologic (NSAID) COX blockade in mice. This is particularly interesting since studies of NSAID-induced asthma have shown changes in mast cell activity,[30,31] although a

link between such increased activity and the reduction of prostanoid production has never been suggested.

Interestingly, in a study using sensitized COX-2 deficient (−/−) and wild-type mice treated with the COX-2 inhibitor nimesulide, Nakata et al[22] did not find changes in airway hyperreactivity in these mice in comparison to untreated sensitized mice. As suggested in a previous study that employed a similar experimental approach,[19] the lack of altered hyperreactivity in knockout mice may be accounted for by the development of compensatory mechanisms in the mutant animals. In addition, the manner in which Nakata et al blocked COX-2 activity in their study might have interfered with the ability to detect possible respiratory function differences. First, their treatment involved administering the blocking agent 3 times during the airway sensitization protocol, in contrast to our daily administration throughout the entire OVA challenge phase. Second, their drug, nimesulide, is a less selective COX-2 inhibitor than rofecoxib. The extent to which our more aggressive approach to inhibiting COX-2 explains the effect on the airway response remains to be determined, but it seems likely that, in order to mimic a potentially naturally occurring deficiency of this enzyme's regulation, pharmacological inhibition of COX-2 requires a strong, sustained blockade over the course of the challenge. It also appears reasonable to propose that, regardless of the strength of inhibition, there would probably be a different effect on respiratory function in relation to whether COX-2 is inhibited at the sensitization and/ or the challenge phase. Finally, Nakata et al used a rather low maximum methacholine concentration (20 mg/mL) and the C57BL/ 6J mouse strain. Thus, discrepancies between our respiratory function data and theirs may be largely explained by methodological differences.

The scoring system we developed, based on previously published methods,[20] took into account both the number of focal accumulations of inflammatory cells and their size. No statistically significant differences were found between rofecoxib-treated and untreated animals. However, based on 3 sets of data we suggest that there seems to be a trend toward more severe inflammation in the airways of animals in which COX-2 was inhibited. First, the increased inflammation score was consistent in every individual, suggesting that results in a larger study group might achieve statistical significance. Second, there was a correlation between inflammation scores and airway responsiveness to methacholine in the rofecoxib treated animals. This is consistent with a role for inflammation in the development of airway hyperreactivity in the murine model used, and supports the suggestion that the increased bronchial responsiveness upon COX-2 blockade may be caused, at least in part, by a worsening of airway inflammation. Third, it is plausible that the observed increase in mucosal mast cell activity leads to increased inflammation.

Further research is needed to confirm whether pharmacologic COX-2 inhibition affects airway inflammation and to ascertain the mechanisms involved. However, our results indicate that strong, sustained inhibition of COX-2 during allergen challenge leads to altered airway function and suggest that this effect is associated with an increase in underlying inflammation. Our main contribution is to reveal the potential relevance of increased mast cell activity in the observed rofecoxib-driven effects. Finally, our data support the hypothesis that abnormal regulation of COX-2 might occur in the development of allergic asthma.

Acknowledgments

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