

Aspirin-Induced COX-2 Overexpression in Monocytes of Aspirin-Intolerant Patients

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Key Words

Aspirin intolerance · Cyclooxygenase-2 expression · Monocytes

Abstract

Background: We hypothesize that alternate regulation of cyclooxygenase-2 (COX-2) may predispose patients to aspirin-induced exacerbations. Therefore, we want to examine the dynamics of COX-2 up-regulation in whole blood monocytes in the presence and absence of aspirin. **Methods:** COX-2 expression was evaluated by flow cytometry through intracellular staining of whole blood monocytes with anti-COX-2 antibodies. Enzyme up-regulation was monitored after in vitro stimulation with lipopolysaccharide (LPS) and/or aspirin in 19 aspirin-intolerant (AI) patients (8 aspirin-sensitive asthmatics and 11 urticaria-angioedema patients) and 14 healthy controls. **Results:** We found significantly higher COX-2 expression levels after stimulation with LPS and aspirin (mean 78.8, range 44.9–92.3; $p = 0.0002$) in comparison to LPS alone (mean 65.9%, range 33.6–82.6) in AI patients. A comparable, but lower up-regulation was also observed after aspirin stimulation alone (median 2.1%, range 0.5–15.9; $p = 0.004$) in comparison with baseline values (median 1%, range 0.1–5.4). There was no significant difference in COX-2 expression between LPS and aspirin stimulation (mean 61.8%, range 26.8–89.2; $p = 0.09$) and LPS stimulation (mean 55.5%, range 28.1–74.3) nor between aspirin stimula-

tion alone (median 0.5%, range 0–8.6; $p = 0.8$) and baseline values (median 0.4%, range 0–5.4) in healthy control subjects. **Conclusions:** The main finding of this study is that COX-2 appears to be differentially regulated in aspirin-sensitive patients. What is really new is the observation that aspirin and LPS increase COX-2 expression on blood monocytes of AI asthmatics, a finding in contrast with the lack of an effect of the same stimuli on COX-2 expression on monocytes from healthy subjects.

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Introduction

Exposure to aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs) may exacerbate nasal and/or respiratory symptoms in sensitive individuals with asthma or cutaneous symptoms in some patients with chronic urticaria. Urticaria and angioedema may also occur in apparently healthy individuals. This phenomenon is very often present when asthma is accompanied by chronic rhinosinusitis and nasal polyposis. The intolerance may manifest itself in a serious anaphylactoid reaction. The pseudoallergic reaction to aspirin has been accepted since the introduction of the drug in therapy. More than a century later, diagnosis relies on either patient history or provocation testing, with the two often yielding discordant results [1–4]. The majority of patients seem to dem-

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Table 1. Demographic data and clinical characteristics of the 19 AI patients

Patient n	Age	Sex	ASA challenge	Chronic conditions	Offending agent (history)	Symptoms on exposure
1	30	M	positive	none	ASA	urticaria/angioedema
2	42	M	positive	none	ASA	urticaria/angioedema
3	31	M	positive	seasonal rhinoconjunctivitis immunotherapy	ASA	urticaria/angioedema
4	48	F	not performed	none	ASA, diclofenac, piroxicam	urticaria/angioedema
5	62	F	not performed	none	ASA	urticaria/angioedema, anaphylactic shock
6	36	M	not performed	none	ASA	urticaria/angioedema
7	54	F	not performed	asthma	ASA	urticaria/angioedema
8	55	M	not performed	none	ASA	urticaria/angioedema, anaphylactic shock
9	49	M	not performed	none	ASA	urticaria/angioedema
10	21	M	positive	chronic urticaria/angioedema	ASA	urticaria/angioedema exacerbation
11	42	F	not performed	asthma, chronic rhinitis	ASA, ketoprofen	bronchospasm, rhinorrhea
12	63	F	not performed	asthma, polyposis	ASA, paracetamol	bronchospasm
13	64	F	not performed	none	ASA, diclofenac	urticaria/angioedema
14	48	F	not performed	asthma, polyposis	ASA, paracetamol, naproxen, diclofenac	bronchospasm
15	50	F	not performed	asthma	ASA, ketoprofen	bronchospasm
16	31	M	not performed	asthma, polyposis	ASA	bronchospasm
17	49	F	positive	chronic rhinosinusitis, polyposis	ASA, paracetamol	bronchospasm
18	41	F	not performed	asthma, chronic rhinitis	ASA	bronchospasm
19	31	M	not performed	asthma, polyposis	ASA, paracetamol	bronchospasm, anaphylactic shock

onstrate a cross-reaction with other NSAIDs, which share the potential with aspirin to inhibit the cyclooxygenase (COX)-1 enzyme. In contrast, most patients are able to tolerate substances that predominantly affect the COX-2 isoform [1, 5, 6].

Accumulated evidence strongly suggests that the pathogenetic mechanism of aspirin intolerance (AI) involves the dysregulation of eicosanoid formation [7]. The underlying mechanism, however, has not yet been unraveled. This is easily conceivable in view of the complexity of the biochemical pathways involved in the regulation and function of eicosanoids [8–10].

The pathogenesis of AI apparently involves the dysregulation of different steps of eicosanoid formation and function, the final result being an increase in cysteinyl leukotriene (CysLT) release [1, 10–12]. These substances are very potent constrictors of bronchial smooth muscles, causing submucosal edema and acting as chemoattractants for eosinophils [13]. Furthermore, increased susceptibility to the action of CysLT seems to be a feature of AI. Alterations in the expression of the leukotriene receptor CysLT1 and, possibly, yet unknown alterations in the eicosanoid milieu may contribute to the clinical manifestations of the syndrome [14].

To date, prostaglandins are known to be synthesized only by COX. The two COX isoforms, COX-1 and COX-2, are traditionally regarded as housekeeping and inducible

isoforms, respectively, but their role appears to be more complex than previously thought [9]. We hypothesize that an alternate COX-2 regulation may be involved in aspirin-induced exacerbations [15, 16]. However, COX-2 is undetectable in most mammalian tissues under basal conditions, although its expression can be rapidly induced by different inflammatory stimuli from bacterial endotoxins, cytokines, growth factors, tumor promoters and hormones in a number of cell types, including monocytes, endothelial cells and fibroblasts [17]. From this point of view, only controlled dynamic studies of COX-2 expression can better evaluate the role of COX-2 in AI. For this reason, we mimicked the *in vitro* inflammatory response in whole blood monocytes using bacterial lipopolysaccharide (LPS, endotoxin) and examined the dynamics of COX-2 up-regulation in the presence and absence of aspirin.

Materials and Methods

Study Subjects

We assessed the expression of COX-2 in blood monocytes obtained from 19 AI patients and 14 healthy volunteers (mean age: 39 years; 8 women). Since the AI patients represent a heterogeneous group, we included patients with different manifestations of AI. Their characteristics are summarized in table 1. The diagnosis of AI was made on the basis of a strongly positive history of aspirin hypersensitivity and/or a positive provocation test (n = 5). None

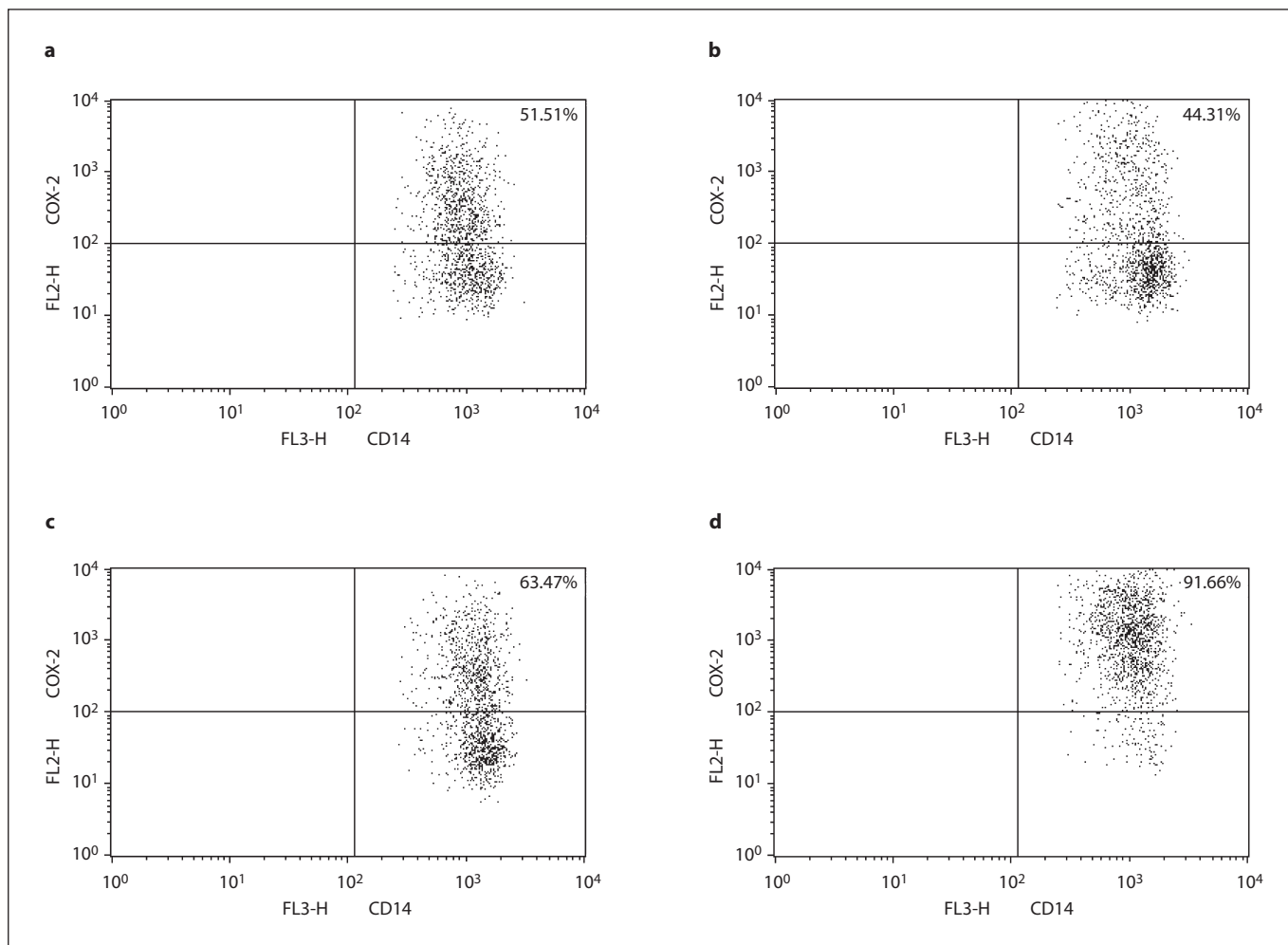


Fig. 1. Representative dot plots of flow-cytometric evaluation of intracellular COX-2 expression in whole blood monocytes in a control subject (**a, b**) and an AI patient with angioedema (**c, d**; patient 2 in table 1) after stimulation with LPS either alone (**a, c**) or combined with aspirin (**b, d**).

of the patients were on oral corticosteroids, immunosuppressive drugs or tricyclic antidepressants at the time of venipuncture or within the recent past. The absence of sensitivity in the control group was confirmed by ingestion of 500 mg of ASA without sequelae. Six milliliters of heparinized venous blood were sampled in the morning. All subjects gave their written consent, and the study was approved by the National Medical Ethics Committee.

Antibodies

For intracellular detection of COX expression, we used PE-conjugated anti-COX-2 mAbs (clone AS67) raised against human recombinant COX-2 protein [18]. Whole blood monocytes were stained with PerCP-conjugated anti-CD14 mAbs. Negative isotype-matched control PE-conjugated mAbs were included in every experiment. All mAbs were purchased from BD Biosciences (San Jose, Calif., USA).

Stimulation and Flow-Cytometric Analysis

Whole blood (with heparin anticoagulant) samples (4×1 ml) were incubated for 4 h at 37°C in an incubator with 5% CO_2 either with LPS ($1 \mu\text{g/ml}$) from *Escherichia coli* 026-B6 (Sigma, St. Louis, Mo., USA), LPS ($1 \mu\text{g/ml}$; Sigma) plus 0.5 mM aspirin (Aspegic, Sanofi-Synthelabo, Quetigny, France), 0.5 mM aspirin (Aspegic, Sanofi-Synthelabo) or with an equal volume of saline ($10 \mu\text{l}$ of 0.9% NaCl; Braun Melsungen, Melsungen, Germany) as a control baseline sample. After 4 h of stimulation, $100\text{-}\mu\text{l}$ aliquots of whole blood were stained with PerCP-conjugated anti-CD14 mAbs for 15 min, lysed for 10 min in 2 ml of $1\times$ FACS lysing solution ($<1.5\%$ formaldehyde and $<5\%$ diethyleneglycol, BD Biosciences), centrifuged at $461 g$ for 5 min and permeabilized for 10 min in 0.5 ml of FACS $1\times$ permeabilizing solution 2 (containing polyoxyethylene sorbitan monolaurate, BD Biosciences). Cells were then washed with PBS supplemented with 1% BSA (BSA/PBS), stained intracellularly with anti-COX-2 mAbs or isotype-matched control mAbs

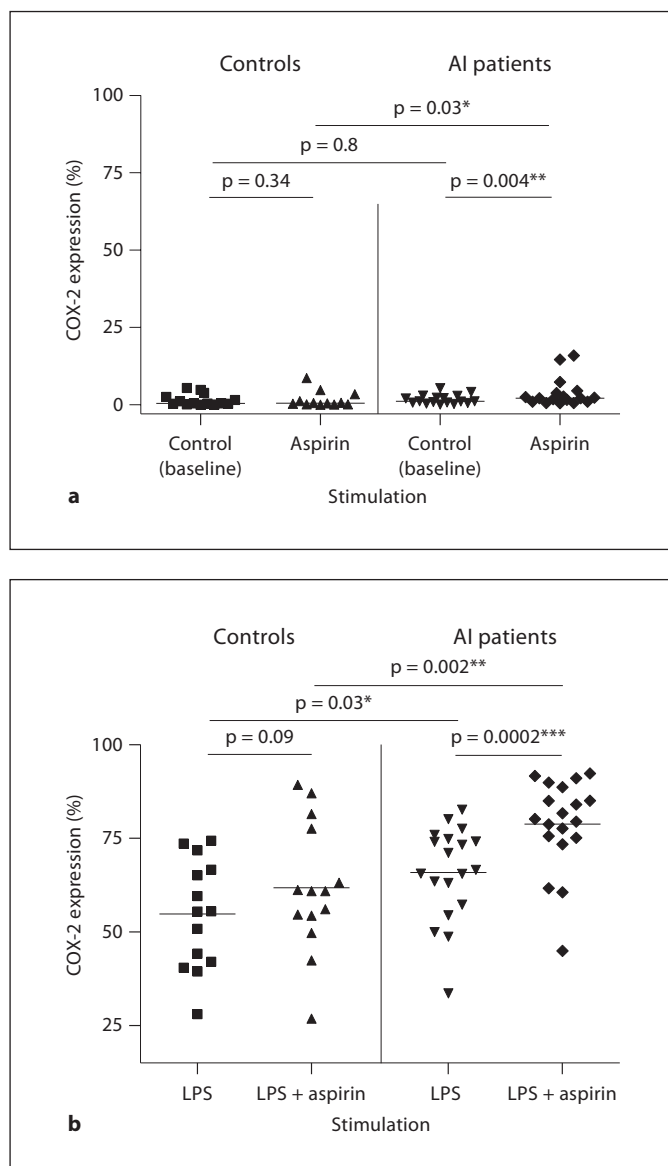


Fig. 2. Summary plot of an overall comparison of COX-2 expression in whole blood monocytes between control subjects (n = 14) and AI patients (**a** n = 17; **b** n = 19) showing baseline expression (control) and after stimulation with aspirin (**a**), and after LPS and LPS plus aspirin stimulation (**b**). Median (**a**) and mean values (**b**) are shown by horizontal lines.

for 30 min, washed in 1% BSA/PBS and fixed in FACS 1× Cell-Fix (1% paraformaldehyde, BD Biosciences). Data were collected with a FACSCalibur (BD Biosciences) and analyzed using CellQuest software. The initial cell gate was defined by side scatter and bright CD14 expression, and at least 2,000 monocytes were analyzed for each probe. The percentage of COX-2 expression was calculated as the number of COX-2- and anti-CD14-positive cells compared to the total amount of CD14-positive monocytes (fig. 1).

Statistical Analyses

The distribution of the data was recalculated by the Shapiro-Wilk test. The statistical significance of COX-2 expression was determined by Student's t test or the Mann-Whitney/Wilcoxon test depending on the type of distribution. The statistical software package GraphPad Prism (version 5.1) was used for all analyses. $p < 0.05$ was considered statistically significant.

Results

The baseline intracellular expression of COX-2 in monocytes was low for all subjects and was comparable between the control group (median 0.4%, range 0–5.4) and the AI patients (median 1%, range 0.1–5.4; $p = 0.34$, Mann-Whitney test; fig. 2a). There was no difference between baseline expression and expression after stimulation with aspirin (median 0.5%, range 0–8.6; $p = 0.8$, Mann-Whitney test; fig. 2a). In contrast, the AI group showed significantly higher, but still low, COX-2 expression after stimulation with aspirin (median 2.1%, range 0.5–15.9; $p = 0.004$, Wilcoxon test) in comparison to baseline values and aspirin-induced COX-2 expression in the control group ($p = 0.03$, Mann-Whitney test; fig. 2a).

Stimulation with LPS induced marked up-regulation of COX-2 expression in monocytes obtained from control subjects and AI patients (mean 55.5 and 65.9%, respectively, range 28.1–74.3 and 33.6–82.6, respectively, fig. 1a, c, 2b), being slightly higher in the AI group ($p = 0.03$, unpaired t test). Surprisingly, we observed much greater COX-2 expression after stimulation with LPS plus aspirin in the AI group compared to LPS stimulation alone (fig. 1c, d, 2b). The mean percentage of COX-2-positive monocytes was 78.8% (range 44.9–92.3). This was significantly higher than stimulation with LPS alone ($p = 0.0002$; paired t test). Moreover, the response of AI patients to LPS plus aspirin stimulation was also significantly higher in comparison to the LPS plus aspirin response in control subjects (mean 61.8%, range 26.8–89.2; $p = 0.002$, unpaired t test; fig. 2b). In contrast, no difference between LPS alone and LPS plus aspirin stimulation was detected in control subjects ($p = 0.09$, paired t test; fig. 1a, b, 2b).

We also analyzed COX-2 expression among the different clinical categories of our patients, divided according to the mode of presentation (respiratory vs. cutaneous symptoms, i.e. aspirin-sensitive asthmatics vs. urticaria-angioedema patients). In both subgroups, we found comparable up-regulation of COX-2 expression after stimulation with LPS (mean 62.9 and 68.1%, respectively, range 33.6–80.1 and 48.7–82.2, respectively), which was signif-

icantly higher and similar in both subgroups after stimulation with LPS plus aspirin (mean 74.6 and 81.8%, respectively, range 44.9–92.3 60.6–91.7; $p = 0.002$ and 0.01 , paired t test, fig. 3).

Discussion

The results of our research suggest an additional pattern of eicosanoid dysregulation contributing to AI. After *in vitro* exposure to aspirin, LPS-stimulated and non-stimulated monocytes from AI patients show a statistically significant increase in COX-2 expression. Our data suggest that aspirin affects the signaling cascade leading to COX-2 up-regulation in these patients. The up-regulation of COX-2 could be a mere manifestation of enhanced inflammation that is triggered or stimulated by other mechanisms. The COX-2 enzyme has been shown, in fact, to be up-regulated by a wide variety of stimuli besides LPS. However, the results obtained for aspirin-induced COX-2 up-regulation in the AI group were remarkably consistent in both LPS-stimulated and native monocytes and, similarly, were unaffected in the control group in both conditions.

The biochemical pathways by which altered COX-2 expression might predispose individuals to AI are not well established. First, previous studies suggest that COX-2 is down-regulated in nasal polyps from aspirin-sensitive asthmatics [15]. Nasal polyps of intolerant patients appear to have lower COX-2 expression after the polyp samples are left at room temperature for 1 h (spontaneous regulation) in comparison to nasal polyp samples from aspirin-tolerant asthmatics [16]. Inadequate COX-2 expression might reduce the production of protective PGE₂, resulting in increased production of pro-inflammatory CysLTs. Hinz et al. [19] suggested that PGE₂ may regulate COX-2 expression in an autocrine manner. Second, when COX-2 predominates, aspirin may induce the production of 5-lipoxygenase via modification of the metabolites formed [20]. COX-2 overexpression does not automatically mean increased synthesis and release of the products, especially because of the presence of aspirin, which is a COX inhibitor. On the other hand, the enzyme can still participate in the formation of other metabolites. Aspirin merely diverts the metabolites formed by COX-2 away from PGH₂ and towards 15-hydroxyeicosatetraenoic acid (15-HETE) [21]. 15-HETE can then be further metabolized by 5-lipoxygenase to the novel 15-epilipoxin [22]. Aspirin-triggered 15-HETE generation in peripheral blood leukocytes was recently suggested as a diagnostic

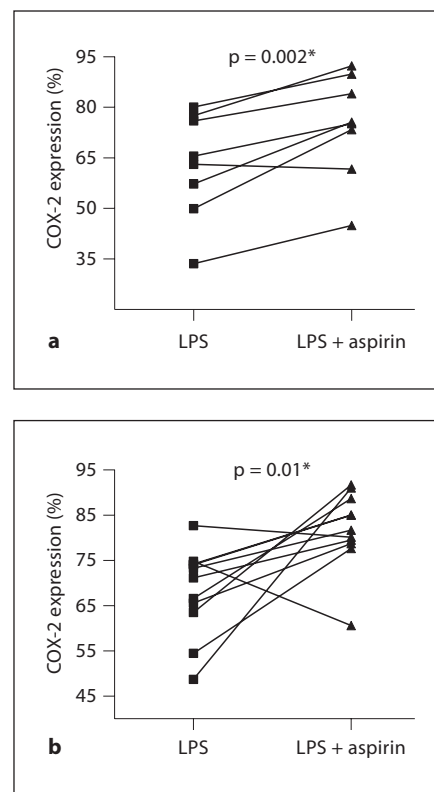


Fig. 3. Summary plot of an overall comparison of COX-2 expression in whole blood monocytes between different clinical categories of AI patients divided according to respiratory versus cutaneous symptoms. **a** Aspirin-sensitive asthmatics ($n = 8$). **b** Urticaria-angioedema group ($n = 11$).

test for AI [23]. Our findings support this second hypothesis and suggest that aspirin may exert its effects by inducing COX-2 expression.

Furthermore, since the changes in COX-2 expression after LPS stimulation have reached statistical significance, the assessment of aspirin-induced COX-2 expression in blood monocytes could be tested as an additional option in the diagnosis of AI. These results clearly indicate that further work is needed to elucidate the effects of aspirin on COX-2 expression.

Our study has several strengths. First, COX-2 is a rapidly inducible enzyme and differences in its expression should ideally be analyzed in a dynamically controlled study rather than static or spontaneous conditions. In all our experiments, we used optimal *in vitro* concentrations of LPS for stimulation that were determined in preliminary studies (data not shown). Second, detection of intracellular COX-2 by flow cytometry suggests further possibilities for quantitative analysis of the protein level in

specific target cells. In addition, there is evidence that COX-2 expression may also be regulated at posttranscriptional checkpoints, indicating limitations in COX-2 mRNA studies. Dixon et al. [24, 25] identified an adenylate- and uridylate-rich mRNA decay element in the 3'-untranslated region of COX-2 messenger RNA that confers posttranscriptional regulation by controlling both mRNA decay and translational efficiency.

There are several limitations to our study. Diagnosis mainly relied on a strongly positive clinical history of aspirin hypersensitivity. However, we obtained COX-2 expression data prospectively and any errors in recall, especially in history and provocation testing, would have tended to attenuate rather than exaggerate an accurate association, often yielding discordant results. We did not include aspirin-tolerant asthmatics in the control group, but the dynamics of COX-2 up-regulation did not differ between aspirin-sensitive asthmatics and the urticaria-angioedema subgroup.

It is interesting to note that a major enhancement of aspirin-induced COX-2 expression was present after in vitro establishment of a proinflammatory environment in the blood sample by LPS addition. In fact, our results indicate the possibility that the discordance between the clinical history of AI and the results of the provocation tests is a consequence of the mechanism of the reaction. Previous studies consistently report low reproducibility for the positive provocation tests in patients with a positive history (21 and 24%) [3, 26]. Based on the results of our

study, we propose the explanation that the large intra-subject variability of responses to NSAID exposure may reflect the changes in the basal inflammatory status of the patients. Perhaps the provocation tests are not able to detect the intolerance because the level of systemic inflammation is low at the time they are performed (usually when patients are stable). In contrast, there is a high probability that an inflammatory state is present when patients take NSAIDs (e.g. to treat fever or pain). As a result, the adverse reaction reappears. Additional clinical studies should further evaluate this hypothesis. On the other hand, the prevalence of intolerance diagnosed by provocation testing is higher than that diagnosed by patient-reported history [1, 2]. Nevertheless, it is difficult to assess the clinical consequences of positive provocation tests in patients with a negative history as it is highly probable that these patients will not be exposed to NSAIDs in the future.

In conclusion, we found aspirin-induced over-expression of COX-2 in blood monocytes of AI patients compared with healthy individuals. Therefore, the main finding of the study is that COX-2 appears to be differentially regulated in aspirin-sensitive patients. What is really new is the observation that aspirin and LPS increase the expression of COX-2 in blood monocytes in AI asthmatics, a finding that contrasts with the lack of effect of the same stimuli on COX-2 expression on monocytes from healthy subjects. These results support the importance of continued investigation of COX-2 expression and related pathways during aspirin-induced exacerbations.

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