Changes in urinary LTE₄ and nasal functions following nasal provocation test with ASA in ASA-tolerant and -intolerant asthmatics

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Summary Aspirin-induced asthma (AIA) is a syndrome characterized by intolerance to aspirin (ASA), nasal polyps and bronchial asthma, the metabolic shift of arachidonic acid towards the lipoxygenase pathway and hyper-production of cysteinyl-leukotrienes (cys-LTs) being the current pathogenetic hypothesis. The research for both sensitive indicators and safe diagnostic tests is still attracting. Aim of the study was to measure changes in urinary LTE₄ excretion and in nasal function (Resistance—Req, and Volume—Vol, assessed by acoustic rhinomanometry (AR)) following a nasal provocation test (NPT) with ASA:LTE₄ measurements have been never previously used to our knowledge for assessing nasal responsiveness to ASA.

Methods: After written consent, 118 mild-to-moderate asthmatics (48 males, mean age 41.8 years ± 11.9 SD, range 25–70 years; basal FEV₁ = 80.1%pred. ± 5.8SD) underwent NPT by nasal instillation of ASA (total maximal dose 25 mg). Spirometry, acoustic rhinomanometry (AR; TM Hood Lab., USA) and urinary LTE₄ (pg/mg creatinine; Cayman Chemical, MI, USA) were measured in baseline and 2 h after the ASA challenge.

Statistics: t-Test between means ± SD, assuming P = 0.05, and linear regression between all variables considered.

Results: In 67 ASA-intolerant asthmatics, FEV₁ did not change significantly following NPT (81.7% pred. ± 5.1 SD in baseline, 80.5% pred. ± 4.1 after NPT, P = ns) even in the presence of a significant decrease of Vol (11.3 cm³ ± 4.1 SD in baseline, 5.9 cm³ ± 4.2 SD after NPT, P = 0.003), a substantial increase of Req (0.88 cmH₂O/l/min ± 0.11 SD in baseline, 2.41 cmH₂O/l/min ± 0.77 after NPT, P = 0.002), and urinary LTE₄ excretion (433.0 pg/mg ± 361.7 in bsln, 858.0 pg/mg ± 471.6 90 min after NPT with L-SA, P = 0.04). NPT did not affect FEV₁ also in 51 ASA-tolerant asthmatics (89.7% pred. ± 6.9 in bsln, 86.6% pred. ± 4.3 after NPT), but in these subjects also Vol (from 14.9 cm³ ± 4.2 SD to 14.6 cm³ ± 3.8 SD), Req (0.38 cmH₂O/l/min ± 0.14 in bsln, 2.12 cmH₂O/l/min ± 0.77 after NPT, P = 0.002), and urinary LTE₄ excretion (163.5 pg/mg ± 115.9 in bsln, 405.0 pg/mg ± 271.6 90 min after NPT with L-SA, P = 0.04).
Introduction

Aspirin-induced asthma (AIA) is a clinical syndrome characterized by acute airway reactions to aspirin (ASA) or other non-steroidal anti-inflammatory drugs (NSAIDS), and nasal polyps. After ingestion of ASA, asthmatic patients with AIA have attacks of bronchospasm that are usually accompanied by symptoms of nasal congestion, mucous secretion and/or skin reactions, such as urticaria. Watery rhinorrhea and nasal obstruction, leading to frequent development of nasal polyps, usually precede the occurrence of asthma and ASA intolerance for months or years. Aggressive nasal polyps and asthma run a protracted course, despite the avoidance of ASA and cross-reacting drugs. AIA is difficult to treat and frequently these patients require therapy with systemic corticosteroids to control symptoms.

The precise mechanism underlying this non-immunological hypersensitivity still remains to be clarified; however, it has been assumed that the power of these pharmacological agents in provoking asthma is related to their activity as inhibitors of cyclooxygenase (COX). The most recent hypothesis is suggesting that the genetic predisposition to the up-regulation of cysteinyl-leukotrienes (cys-LTs) pathway could be related to an overactive expression of LTC4 synthase.

Patients with AIA are also characterized by lower production of prostaglandin E2 (PGE2) rather than ASA-tolerant patients and that this alteration in PGE2 production is probably due to an inadequate COX-2 regulation.

Even in stable clinical conditions, AIA patients have an increased basal production of cys-LTs (such as urinary LTE4). They are also characterized by a significant increase in urinary LTE4 excretion following ASA ingestion and following ASA inhalation: other peculiar features of these patients are their higher levels of urinary LTE4 and LTB4 glucoronide (a chemotactic factor for eosinophils and neutrophils) following the intravenous ASA challenge.

The research for both sensitive indicators and safe diagnostic tests is still attracting. Nasal provocation test (NPT) with lysine-aspirin (L-ASA) has been introduced a few years ago for assessing AIA also in patients with instable asthma: the test has been described as a highly specific and sensitive one, but negative results do not exclude possible intolerance to ASA. More recently, the acoustic rhinomanometry (AR) has been suggested as the most helpful diagnostic procedure for NPT with L-ASA, its use being less limited than the anterior rhinomanometry by the presence of nasal polyps and nasal obstructions.

Measurements of urinary LTE4 collected before and after the challenge were never associated to our knowledge to the NPT test up to now.

Aim of the present study was to measure changes in urinary LTE4 and nasal function assessed by AR induced in mild-to-moderate asthmatics by a nasal challenge with ASA.

Materials and methods

Subjects and design

After written consent, 121 mild to moderate ever non-smoker asthmatics were initially screened to undergo a NPT with L-ASA. Patients performed NPT for a suspicious of AIA and/or for a history of nasal polyps, or chronic rhinitis. As the test should not be performed in the presence of the total obstruction of at least one nostril, the results of the AR led to exclude three patients. Thus, 118/121 subjects entered the study (48 males, mean age $41.8 \pm 11.9$ years, range 25–70 years; basal FEV1 = $80.1\%$ pred. $\pm 5.8\%$) and performed the NPT with L-ASA after having stopped their medications (oral antihistamines and nasal corticosteroids for 7 days, and nasal inhaled ant-H1 agents for at
least 48 h before NPT). Patients stopped any concomitant leukotriene receptor antagonists during a 2-week washout period prior to the challenge. Patients were also required to withhold any short-acting \( \beta_2 \)-agonists and long-acting \( \beta_2 \)-agonists for at least 6 and 48 h, respectively, prior to NPT.

None of the subjects were receiving systemic corticosteroids or a 5-lipoxygenase inhibitor, and 99 out of the 118 asthmatic patients were using inhaled steroids (median 500 \( \mu \)g equivalent to BDP/day; range 400–1000 \( \mu \)g), in 56 cases associated with long-acting \( \beta_2 \)-agonists. No patients reported signs and symptoms of any infection of upper or lower airways in the 4 weeks prior to the study.

**Acoustic rhinomanometry**

Each patient performed the NPT according to the method described by Casadevall and co-workers by means of the AR\(^{18}\). This method consists in the measurement of acoustic reflections from the nasal cavity of a sound pulse created by a spark in a sound tube connected to the nasal cavity via a nosepiece. Unlike conventional rhinomanometry, AR does not require generation of nasal flow, and therefore its use is less limited by the presence of nasal polyps and nasal obstruction. The response was evaluated by the Eccovision Acoustic Rhinomanometry System (TM Hood Laboratories, USA) with the measurement of\(^{22}\):

1. calculated resistance, based on a tube with the same area and laminar flow (\( R_{eq} \), mmH\(_2\)O/l/min);
2. the total volume of the nostrils (Vol, cm\(^3\)) represents the nasal cavity volume in the analysis segment;
3. the minimal cross-sectional area (cm\(^2\));
4. its distance from the nosepiece (cm);

Rhinomanometric measurements were performed in an isolated space while the subject was in apnoea after a non-forced expiration. The rhinomanometer was calibrated daily with a calibration tube provided by the manufacturer. The analysis of data was performed using the Kwikstat program (TM Texasoft).

**Nasal provocation test with L-ASA**

Baseline nasal function was measured with AR, while the subjects were in a sitting position, then 80\( \mu \)l of L-ASA solution (180 mg/ml L-ASA) was applied locally through nose droplets on the inferior nasal concha in both nostrils. The total deposited dose of L-ASA was equivalent to 25 mg of acetylsalicylic acid. AR was then performed bilaterally every 10 min for the next 2 h.

L-ASA was prepared freshly each day by dissolving crystalline L-ASA in 0.9% sodium chloride to produce a solution containing 180 mg/ml.

NPT was considered positive when: nasal resistance increased more than 40% in at least one nostril as compared with the corresponding baseline value; when the volume of one nostril decreased more than 10% from baseline; both parameters sustained for at least two consecutive measurements, and were accompanied by clinical symptoms persisting at least 30 min. The dose of ASA, the duration of the observation period, and criteria for positivity of the test were established on the basis of previous experiments.\(^{16-18}\)

Simultaneously, pulmonary function (FEV\(_1\); forced expiratory volume in 1 s) was measured by using a computerized pneumotachograph (Masterlab, Jaeger). We considered the maximal fall for \( R_{eq} \), Vol and FEV\(_1\) observed in the 2 h following the NPT.

**Urinary LTE\(_4\)**

Urine samples were obtained before NPT with L-ASA and 2 h after the nasal instillation of L-ASA. The time for urine collection was based on a previous measure of urinary LTE\(_4\) after inhalation challenge.\(^{16}\)

Urinary LTE\(_4\) were measured by enzyme immunoassay (ACE\(^{TM}\) Competitive Enzyme Immunoassay, Cayman Chemical, Ann Arbor, Mich, USA), as reported by Pradelles et al.\(^{23}\)

This assay is based on the competition between LTE\(_4\) and an LTE\(_4\)–acyethylcholinesterase (AChE) conjugate (LTE\(_4\) tracer) for a limited amount of LTE\(_4\) antiserum. Because the concentration of the LTE\(_4\) tracer is held constant while the concentration of LTE\(_4\) varies, the amount of LTE\(_4\) tracer that is able to bind to the LTE\(_4\) antiserum will be inversely proportional to the concentration of LTE\(_4\) in the well. This antibody–LTE\(_4\) complex blinds to a mouse monoclonal anti-rabbit IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman’s reagent (which contains the substrate to AChE) is added to the well. This reagent consists of acetylthiocholine and 5,5’-dithio-bis-(2-nitrobenzoic acid). Hydrolysis of acetylthiocholine by AChE produces thiocholine. The non-enzymatic reaction of thiocholine with 5,5’-dithio-bis-(2-nitrobenzoic
acid) produces 5-thio-2-nitrobenzoic acid, which has a strong absorbance at 412 nm. AChE has several advantages over other enzymes commonly used for enzyme immunoassays. Unlike horseradish peroxidase, AChE does not self-inactivate during turnover. In addition, the enzyme is highly stable under the assay conditions, has a wide pH range (pH 5–10), and is not inhibited by common buffer salts and preservatives. The product of this enzymatic reaction has a distinct yellow colour and absorbs strongly at 412 nm. The intensity of this colour, determined spectrophotometrically, is proportional to the amount of LTE₄ tracer bound to the well, which is inversely proportional to the amount of free LTE₄ present in the well. LTE₄ was measured by enzyme immunoassay on all samples according to the manufacturers’ instructions and expressed in pg/mg creatinine (pg/mg).

**Statistical analysis**

Mean values ± SD obtained before and after NPT for each variable were compared by t-test, and \( P < 0.05 \) was assumed as the lowest limit for the statistical significance.

Linear regression was calculated for all variables considered.

**Results**

In 67 subjects (the ASA-intolerant asthmatics), NPT was discriminant for ASA intolerance: FEV₁ did not change significantly (81.7% pred. ± 5.1 SD in baseline, 80.5% pred. ± 4.1 after NPT, \( P = \text{ns} \)) but a substantial increase of Req (0.88 cmH₂O/l/min ± 0.11 SD in baseline, 2.41 cmH₂O/l/min ± 0.77 after NPT, \( P = 0.002 \)) and a corresponding significant decrease of total volume (11.3 cm³ ± 4.1 SD in baseline, 5.9 cm³ ± 4.2 SD after NPT, \( P = 0.003 \)) were systematically recorded.

When compared to the corresponding basal values, Req and Vol changes were discriminant for ASA intolerance according to the criteria described by Milewski et al.¹⁹ The diagnostic value of nasal changes following NPT was further emphasized by the changes observed in urinary LTE₄ in the same group of patients (433.0 pg/mg ± 361.7 in baseline, 858.0 pg/mg ± 471.6 after 2h since the NPT with L-SA, \( P = 0.04 \)) (Fig. 1).

In the remaining 51 subjects (such as the ASA-tolerant asthmatics), NPT did not affect FEV₁ at all (89.7% pred. ± 6.9 in bsln, 86.6% pred. ± 4.3 after NPT), but in these subjects both Req (0.38 cmH₂O/l/min ± 0.14 in bsln, 0.26 cmH₂O/l/min ± 0.2 after NPT, \( P = \text{ns} \)), and Vol (from 14.9 cm³ ± 4.2 SD to 14.6 cm³ ± 3.8 SD) also unchanged. LTE₄ excretion was not affected by NPT (333.1 pg/mg ± 202.8 in baseline, 318.0 pg/mg ± 198.7 after NPT, \( P = \text{ns} \)) and it represents a further confirmation of ASA tolerance for these subjects (Fig. 2).

While unrelated to age and basal FEV₁ (\( r = -0.05 \) and \( r = 0.01 \), respectively), pre-NPT LTE₄ proved directly related to pre-NPT Req (\( r = 0.54 \) and...
inversely (more strictly) related to pre-NPT Vol values \((r = -0.71)\). Post-NPT LTE\(_4\) proved unrelated to corresponding post-NPT Req \((r = 0.19)\) even though still inversely related to post-NPT Vol values \((r = -0.59)\) (Fig. 3a–d).

NPT proved well tolerated in the whole population: no systemic or skin reaction was registered, and no episode of bronchospasm occurred.

**Discussion**

AIA develops according to a clinical pattern characterized by a sequence of symptoms\(^3\): firstly, persistent rhinitis, then asthma, ASA intolerance, and finally nasal polyposis appear. After the occurrence of rhinitis, it is important to precise AIA because such a particular asthma has to be considered a “difficult” asthma which frequently requires systemic steroids to control symptoms.

There is no in vitro test for identification of ASA intolerance, and the diagnosis can be only confirmed by oral\(^{24}\) or by inhalation\(^{25,26}\) ASA provocation test. Both these procedures have several limits: while the oral test can sometimes precipitate a severe asthmatic reaction, the inhalation of increasing concentrations of L-ASA solution is considered as unsuitable for patients whose asthma is not in clinical remission and whose FEV\(_1\) is lower than 70% of predicted value.

At present, NPT is regarded as the method of choice for assessing AIA in ASA-intolerant subjects by means of anterior rhinomanometry,\(^{19}\) or AR,\(^{20,21}\) or by measuring peak nasal inspiratory flow, which has been shown to offer a greater reproducibility than AR.\(^{27}\)

In the present study, NPT confirmed as a test simple and rapid to perform, and highly safe for diagnosing AIA; it should then represent the method of choice to check the hypersensitivity to ASA which manifested by symptoms of upper
respiratory tract only. In particular, different from the older and usual diagnostic approach, NPT, when based on the assessment of ASA-induced acoustic rhinomanometrical changes, consented the absolute protection of lung function. For these reasons, this novel procedure should be used more widely as the first-line diagnostic approach to the diagnosis of hypersensitivity to ASA also in patients with instability of the bronchial tree, and when bronchial or oral provocation test with ASA cannot be carried out for safety reasons.

Several observations are supporting the hypothesis that LTs may mediate AIA; in particular it has been assessed a mean fourfold increase in urinary LTE4 levels 3–6 h following ASA ingestion, and after inhalation of L-ASA. Moreover, the severity of respiratory reactions during oral ASA challenge was associated with the degree of elevation of baseline LTE4 expression, thus suggesting that asthmatics with ASA-sensitive respiratory disorders have a spectrum of reactions in which leukotrienes seem to play a crucial role. Despite these observations, changes in urinary LTE4 following NPT with ASA were never previously investigated in ASA-tolerant and -intolerant asthmatics.

As reported in previous studies, also the basal elevation of urinary LTE4 excretion seems to characterize and discriminate ASA-sensitive asthmatics from non-sensitive controls. Urinary LTE4 excretion is significantly enhanced after the simple nasal instillation of L-ASA, thus emphasizing the great diagnostic value of LTE4 measurements even in the absence of systemic reactions. Since even at baseline the cysLTs production is enhanced in ASA-intolerant asthmatics, anti-leukotriene therapy could be an option for these type of asthmatics. This condition in fact proved mainly sensitive to a therapeutic course with Montelukast, a specific LT1 receptor antagonist. Montelukast improved also nasal function and nasal response to ASA in ASA-sensitive asthmatics.

Moreover, despite age and lung function (FEV1), the extent of basal LTE4 urinary excretion seems clearly related to basal (such as pre-NPT) nasal function, in particular to nasal volume. The crucial role of LTE4 production (which is already exhaled in baseline) and of nasal structural involvement (i.e. nasal hyperresponsiveness) can be regarded as a substantial contribution to the early definition of the biological and functional pattern of ASA intolerance.

Conclusions

Data of the present study further support the diagnostic relevance of basal LTE4 measurements and of nasal investigation in patients suspected of ASA intolerance, thus emphasizing the intrinsic predisposition to LTs-mediated reactivity to ASA. Even though further studies are needed to confirm present pilot data, NPT with L-ASA, when implemented with the AR and urinary LTE4 measures, confirms as a test absolutely safe and discriminant for patients suspected of ASA intolerance, and represents a suitable diagnostic procedure in the absence of meaningful bronchial obstruction.

References


