

REVIEW

Direct or indirect stimuli for bronchial challenge testing: what is the relevance for asthma epidemiology?

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Introduction

The prevalence of asthma has increased substantially over the last several decades. In the industrialized world, it is now the most common chronic disease in childhood and early adolescence [1–3]. Asthma is characterized by reversible airway obstruction and bronchial hyperresponsiveness (BHR), defined as excessive airway narrowing, with symptoms of wheeze, shortness of breath, or cough in response to non-specific stimuli such as cold air, exercise, and tobacco smoke.

Epidemiological studies of asthma aim to estimate the burden of this disease and risk factors for its development. These studies may provide targets for prevention either in the general population, or in subgroups. Furthermore, they may provide tools to identify subjects in an early phase of the disease, preferably prior to the development of symptoms.

In recent years, the assessment of BHR has become common practice in epidemiological studies. Steering committees of the European Community Respiratory Health Survey and the International Study on Allergy and Asthma in Childhood (ISAAC) have approved standardization of bronchial challenge tests for methacholine and hypertonic saline (HS). The worldwide use of these protocols allows international comparison of results. This may provide new insights into genetic and ecological risk factors, as well as the significance of BHR in the pathogenesis and diagnosis of asthma in the community.

The pathological features that underlie BHR are presumably bronchial inflammation and airway remodelling. The assessment of BHR in the laboratory entails inhalation of a stimulus that causes bronchoconstriction in people with asthma, but causes little or no response in people without asthma. Different stimuli are being used to test for BHR, and each may constitute a piece of the jigsaw puzzle on the pathophysiology of BHR and asthma. In general terms, stimuli are categorized as either 'directly' or 'indirectly' acting. Histamine and methacholine are commonly used direct-acting stimuli that cause bronchoconstriction by directly stimulating the contraction of airway smooth muscle cells [4]. In contrast, indirect stimuli cause bronchoconstriction by mediators released from intermediary cells, like inflammatory cells, epithelial cells, or nerves [5, 6]. Common examples of

indirect stimuli are exercise, hyper- and hypotonic solutions, cold or dry air, and adenosine.

The aim of this paper is to review BHR to the indirect stimuli adenosine and HS with respect to their mechanism of action, and their relationship to eosinophilic bronchial inflammation. Subsequently, we will discuss their value in epidemiological studies. We have chosen to focus on adenosine and HS as bronchoconstrictive stimuli because adenosine has been studied extensively in adults in recent years, and HS has been adopted as the stimulus for measuring BHR by the steering committee of the ISAAC and is currently widely used in clinical and epidemiological studies [7].

Mechanism of bronchoconstriction

Methacholine and histamine

Methacholine and histamine have been used in bronchial challenge testing for several decades. For both types of challenge tests, there are standardized protocols available that allow comparison of BHR with these stimuli over time and between populations [4]. Methacholine and histamine are both pharmacological stimuli that cause bronchoconstriction by directly activating contraction of bronchial smooth muscle cells after binding to cholinergic receptors [8–10], and additionally to histamine receptors in case of histamine inhalation [8, 11–13].

People with asthma show an increased bronchoconstrictive response, i.e. they respond with more pronounced airway narrowing with a similar dose of inhaled stimulus, compared to healthy, non-asthmatic subjects. The physiological basis of this increased contractility remains fundamentally unresolved.

Adenosine and hypertonic saline (HS)

Bronchoconstriction after inhalation of adenosine or hypertonic solutions is considered to be mainly due to the action of mediators that are released from intermediary cells, and to a much lesser extent to direct activation of cholinergic nerve fibres [5]. The principal difference between both stimuli is that adenosine is a pharmacological stimulus that exerts its action on mast cells by binding to specific adenosine receptors of the A2B type [14–16], whereas hyperosmolarity is considered to be a physical stimulus that is presumed to provoke bronchoconstriction in a less specific way.

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Adenosine is administered in an aqueous solution as adenosine-5'-mono-phosphate (AMP). After inhalation, this is converted rapidly to adenosine by the ubiquitous enzyme 5'-nucleotidase. Although the exact mechanism underlying the effects of AMP on the airway wall has not yet been fully elucidated, there is mounting evidence that the release of inflammatory mediators from mast cells plays a crucial role.

Firstly, adenosine enhances the release of a variety of inflammatory mediators such as histamine, prostaglandins, leukotrienes, and IL-8 from human mast cells *'in vitro'* [17–20]. Secondly, bronchial challenge with adenosine or AMP results in an immediate increase of histamine [21, 22], and (mast cell specific) tryptase in bronchoalveolar lavage (BAL) fluid [22]. Thirdly, adenosine-induced bronchoconstriction is more than 80% inhibited by the selective histamine receptor antagonists terfenadine and astemizole [23, 24]. Moreover, mast cell-stabilizing agents, nedocromil and cromoglycate, also attenuate AMP-induced bronchoconstriction [25–27].

Bronchial challenge with HS has been developed as a surrogate measure for exercise-induced bronchoconstriction, based on the assumption that the latter is mainly caused by an increase in the osmolarity of airway lining fluid due to the evaporation of water caused by hyperventilation during exercise [28]. In comparison with exercise challenge testing, the use of an inhaled hyperosmotic solution as a challenge agent has the advantage of improved test standardization, and feasibility in subjects who are not able to perform extraneous exercise. Indeed, BHR to HS is strongly associated with the presence of exercise-induced asthma [29–31].

The degree of hyperosmolarity is the main determinant of HS-induced bronchoconstriction: the more hypertonic, the more potent it is to induce airway obstruction [32, 33]. The mechanism by which HS causes bronchoconstriction has not yet been elucidated in detail, but there is mounting evidence for the involvement of mast cells. *In vitro*, hypertonic stimulation causes histamine release from human mast cells and basophils [34, 35]. Furthermore, HS-induced bronchoconstriction is attenuated by pre-medication with H1-receptor blockers terfenadine or clemastine [36–39]. Similar attenuation was observed after pre-medication with mast cell-stabilizing agents nedocromil or sodium cromoglycate [40, 41].

Thus, the mechanisms underlying adenosine- and HS-induced bronchoconstriction appear to have strong similarities. However, it is unlikely that they work through the same pathways because prior exposure to one of these agents does not induce refractoriness to the effect of subsequent exposure to the other [41], whereas each does induce refractoriness to re-exposure to itself [42–44]. Furthermore, it has been shown that pre-medication by the anti-histamine terfenadine reduces bronchoconstriction by adenosine (>80%) [23, 24] more strongly than by HS (>65%) [38]. These observations strongly suggest dissimilarities in the mechanism of action or sources of mediators released. For both stimuli, cholinergic stimulation contributes to the bronchoconstriction, although this effect is smaller than the effect of histamine release. Pre-medication with the anti-cholinergic drug ipratropium bromide attenuates the response by a factor 2–2.5-fold to both adenosine [45–47].

There is growing evidence that leukotrienes also play a role in the mechanism of adenosine-induced bronchoconstriction. Van Schoor et al. [48] found increased urinary leukotriene E₄

(LTE₄) secretion after both adenosine- and exercise-induced bronchoconstriction; leukotriene B₄ production only increased after exercise-induced bronchoconstriction. In this study, pre-medication with the lipoxygenase inhibitor ABT-761 protected more strongly against exercise-induced bronchoconstriction, and decreased leukotriene excretion. Other studies have confirmed the role of leukotrienes in adenosine-induced bronchoconstriction by showing attenuation after pre-medication with the leukotriene receptor antagonist montelukast [49, 50].

The role of leukotrienes in HS-induced bronchoconstriction has not yet been studied. The role of leukotrienes in the mechanism of exercise-induced bronchoconstriction has been shown in a number of studies. Bronchoconstriction in response to exercise results in LTE₄ excretion [51], and is attenuated after pre-medication with leukotriene receptor antagonist montelukast [52], and the lipoxygenase inhibitor ABT-761 [53]. Assuming that hyperosmolarity is the common mechanism in exercise and HS-induced bronchoconstriction, the above observations suggest a role for leukotrienes in both bronchoconstricting stimuli.

In conclusion, adenosine and HS inhalation cause bronchoconstriction due to mast cell mediator release and, to a lesser extent, due to cholinergic activation. Notwithstanding their similarity in the underlying mechanism of mast cell stimulation, there are mechanistic differences as well. The role of mast cell-derived histamine seems greater in adenosine-induced bronchoconstriction, since antihistaminic pre-medication results in a greater reduction of the response. Leukotriene release contributes to adenosine-induced bronchoconstriction, but its role needs to be confirmed in HS-induced bronchoconstriction. There may be differences in the relative contribution of leukotrienes, and in the leukotriene profile.

BHR as a marker of bronchial inflammation

Bronchial inflammation in asthma is characterized by an increased number and activation of mast cells and eosinophils [54–61]. Both cell types correlate with clinical severity [62], and decrease after corticosteroid treatment [63–65]. There is evidence of a two-way interaction between mast cells and eosinophils. It is well known that mast cell mediators stimulate the release of mediators from eosinophils [66–68]. Recent studies have also shown evidence of mast cell activation by eosinophils, suggesting a role of mast cells in the late phase of an allergic reaction, independent of IgE stimulation [69, 70]. *In vivo*, a high level of sputum tryptase is associated with a higher number of sputum eosinophils and eosinophilic cationic protein (ECP), both in people with asthma [71, 72], and in patients with eosinophilic chronic airway obstruction [73].

As previously described, adenosine- and HS-induced bronchoconstriction in individuals with asthma involves degranulation of activated mast cells, resulting in an increased release of bronchoconstrictive mediators such as histamine and leukotrienes. Assuming a relationship between mast cells and eosinophils, bronchial responsiveness to these indirect stimuli indirectly reflects bronchial eosinophilic inflammation. This hypothesis is supported by the observation that

interventions directed against airway inflammation have a greater effect on bronchial responsiveness to indirect agonists than to direct agonists. For example, allergen avoidance, achieved by living at high altitude, reduces BHR to AMP, but not BHR to methacholine [74]. Furthermore, anti-inflammatory treatment with corticosteroids causes a greater reduction in responsiveness to adenosine than to methacholine [75–79]. Bronchial responsiveness to HS is also reduced after corticosteroid treatment [80–82], although less pronounced than AMP responsiveness.

Compared with methacholine hyperresponsiveness, AMP hyperresponsiveness shows a stronger association with blood and sputum eosinophils [83, 84]. Moreover, van den Berge et al. [79] have shown that the reduction in sputum inflammatory markers after corticosteroid treatment was more strongly associated with a simultaneous reduction in bronchial responsiveness to AMP than to methacholine. Conversely, both in this study among asthma patients and in the general population, methacholine responsiveness was more closely related to a lower pre-challenge level of forced expiratory volume in 1 s [83]. This might suggest that BHR to methacholine is more strongly determined by the long-standing sequelae of the inflammatory process in the bronchi instead of acute inflammatory events. This is supported by the observation that BHR to methacholine shows initial improvement immediately after the start of inhaled steroid treatment, but continues to improve further over a period of several months [85, 86]. Ward et al. [86] showed that basement membrane thickness reduced after 12 months steroid treatment, whereas the maximum change in BAL inflammatory cells and lung function already occurred within 3 months of treatment.

Conversely, AMP-induced responsiveness might reflect to a larger extent acute inflammatory changes, or the propensity to mediator release of eosinophils in the airway wall. The latter is in agreement with the observation that AMP responsiveness is a better predictor for the development of upper respiratory tract symptoms than methacholine responsiveness [87].

Studies on the relationship of hyperosmolarity-induced bronchoconstriction with eosinophilic inflammation are scarce. Indeed, BHR to HS correlates better with blood eosinophil counts than BHR to methacholine. Gibson et al. [62] found a positive correlation between the degree of responsiveness and sputum eosinophil numbers in asthmatic patients. In another study among children, BHR to HS was associated with a higher number of sputum eosinophils, but only if asthmatic symptoms were present as well [88]. In a Dutch population of 8–13-year-old schoolchildren, we also observed higher blood eosinophil numbers and serum ECP levels in children with both asthmatic symptoms and BHR to HS in comparison with children with asymptomatic BHR or without BHR. In asymptomatic children with BHR, we only observed a higher serum ECP in atopic children (submitted).

Significance of BHR in epidemiology

Epidemiological studies aim to assess the prevalence and severity of disease, to identify preventable risk factors, and

observe the disease in its early phase [89]. Asthma is a disease characterized by variation over time in symptoms, lung function, and pathology. Epidemiological studies do not usually have the opportunity to detect this variability over time. Hence, in epidemiological surveys, asthma diagnosis is mainly based upon reports of symptoms or a doctor's diagnosis of asthma. The role of the measurement of BHR in this context is discussed below.

BHR in the detection of asthma

There is some controversy about the role of measurement of BHR for identifying asthma in epidemiological studies. Some investigators have sought to assess the sensitivity and specificity of BHR for the detection of asthma. However, there are important theoretical limitations to this form of analysis. There is no criterion or 'gold standard' to establish the diagnosis of asthma. A few studies have evaluated BHR using 'a doctor's diagnosis of asthma' as the gold standard. These studies have shown poor agreement between these two measures and have concluded that BHR has a low sensitivity for detecting asthma in the general population. This was irrespective of whether the bronchoconstrictive stimulus was methacholine [90–92], adenosine [92], or HS [93–95].

However, there are major problems with the assumption that a reported 'doctor's diagnosis of asthma' fulfils the criteria of a 'gold standard' for asthma, against which sensitivity and specificity should be assessed. The diagnosis of asthma is usually made by a primary-care physician (General Practitioner) on the basis of the patient's report of symptoms and, possibly, the observation of signs of wheezing if the patient presents during an acute exacerbation. It is unusual for the diagnosis to be made on the basis of observed variability in symptoms or lung function over time. The likelihood that a patient will acquire a diagnosis of asthma is strongly influenced by the doctor's knowledge and understanding about asthma, and the patient's accessibility to the health-care system. Since doctors usually diagnose asthma on the basis of symptoms reported to them, it is hardly surprising that there is a relatively good agreement between patients' reports of symptoms and having been diagnosed with asthma [96].

A diagnosis of asthma can only be made with certainty by monitoring the symptoms and lung function over a period of time. The validity of a single-point measurement of any characteristic, such as symptoms, lung function, BHR, or a reported diagnosis, for the detection of asthma defined in this way has not yet been assessed.

Heterogeneity of BHR

Bronchial challenge tests convey additional information on the pathology, therapeutic response, and prognosis of asthma. Different stimuli address different aspects of airway pathology. As noted above, bronchial challenge tests with adenosine and HS may be more specific for bronchial inflammation with involvement of mast cells and eosinophils, irrespective of the clinical diagnosis. It has been shown that, in contrast to methacholine responsiveness, responsiveness to adenosine is related to the presence of atopy, both in asthmatics [83, 97], and in non-asthmatics [98].

Furthermore, it distinguishes children with asthma from those with other airway diseases (cystic fibrosis, bronchiectasis, and bronchiolitis) [99]. As mast cells are not the main cell type involved in the pathology of chronic obstructive pulmonary disease (COPD), AMP responsiveness may be expected to be lower in COPD compared with atopic asthma. This has been confirmed in non- and ex-smokers. Smoking enhances AMP responsiveness in people with asthma [100], and in subjects without rhinitis [101]. These studies show that BHR to indirect stimuli may provide a non-invasive tool to study the pathogenetic effects of environmental exposures on bronchial inflammation.

BHR as a predictor of asthma

The presence of BHR to specific stimuli has prognostic significance for the development of symptoms, the response to treatment, and the course of the disease. BHR to histamine or methacholine has been shown predict the development of asthmatic symptoms, both in children [102–106] and in adults [107, 108]. Furthermore, BHR predicts impaired lung function growth in childhood [109–111] and accelerated lung function decline in adults [112, 113].

The risk of developing symptoms increases if higher blood eosinophil numbers co-exist [114], suggesting a role of pre-existent eosinophilic activation. This is in agreement with a recent observation of our group that AMP responsiveness was a stronger predictor for the subsequent development of allergic symptoms than methacholine responsiveness, in newly appointed laboratory animal workers and bakers [87]. However, this was a small study with only 12 subjects developing symptoms. Thus, further studies have to replicate these observations.

Furthermore, BHR is of prognostic value in subjects with asthmatic symptoms. In symptomatic subjects, those with BHR are more likely to have persisting symptoms [103, 108, 111], and BHR increases over time [115]. Remission of symptoms may occur [116], although it is more likely to happen in people with mild BHR [117]. However, remission of symptoms does not necessarily imply remission of the underlying inflammation [118]. Occupational studies have investigated the remission of asthma in more detail. Even after complete withdrawal from occupational exposure, remission of BHR to methacholine occurs in only 25% of the patients with occupational asthma [119, 120]. So far, no data have been published on the prognostic implications of changes in BHR to indirect stimuli in symptomatic subjects. Presumably, this will be affected strongly by the response to anti-inflammatory treatment.

The relative effect of two anti-inflammatory modalities, inhaled corticosteroids and allergen avoidance, on BHR to direct and indirect agonists has been assessed. There is consistent evidence that, in patients with asthma, corticosteroid treatment decreases BHR to AMP [76, 77] and HS [80–82] more strongly than BHR to methacholine.

Allergen avoidance had a greater effect on AMP responsiveness than methacholine responsiveness in one study [121], whereas an equal reduction of both AMP and methacholine responsiveness was observed by Grootendorst et al. [122]. However, in the latter study corticosteroid therapy was

continued, which may have decreased the effect of allergen avoidance on AMP responsiveness.

Concluding remarks

There is strong evidence that BHR to AMP is a better marker than BHR to methacholine for the involvement of mast cells and eosinophils in asthma. BHR to HS may also be a good marker for mast cell and eosinophilic airway inflammation, but the evidence is less strong. It should be emphasized that this inference is based upon clinical studies among asthmatic patients, and the relationship with bronchial inflammation in non-asthmatics is largely unknown. Moreover, there are no data on the relationship between eosinophilic inflammation and variation in bronchial responsiveness to AMP below the arbitrary cut-off level for a positive test.

It is plausible that even low levels of responsiveness to an indirect stimulus are indicative of the presence of abnormal inflammation and hence markers for the risk of subsequent asthma. If this is proved to be correct, it would have important implications for the screening and prevention of asthma.

We propose that the use of an indirect stimulus in epidemiological studies will yield a more specific risk profile for eosinophilic asthma, which may guide the development of more specific and effective prevention strategies. However, the disadvantage of using a more specific stimulus is that it will probably be less sensitive. So far this has not been formally tested. If this is the case, it will limit its value as a single bronchial challenge test for use in population studies. Since including more than one bronchial challenge test is not practical in large-scale studies, the selection of an appropriate challenge agent for testing BHR will depend on the specific hypotheses to be tested. The evidence presented here suggests that methacholine responsiveness is a better marker of the chronic sequelae of bronchial inflammation, whereas AMP and HS responsiveness more accurately reflect acute inflammatory changes.

Assessing BHR in epidemiological studies should not primarily aim to detect subjects with asthma. Instead, BHR should be regarded as a tool to examine the heterogeneity of asthma in populations. Specific stimuli provide additional information on the underlying pathology, and its prognosis. Furthermore, they may provide insights into the pathological consequences of a variety of environmental conditions, such as occupational exposures, air pollution, or smoking. The development of bronchial challenge tests using stimuli that address other pathological features of airway obstruction will assist in the search for environmental factors that play a role in asthma, and the evaluation of environmental prevention measures.

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