Optimizing and evaluation of a methacholine provocation test

with application in occupational research

BY

BRITT-MARIE SUNDBLAD
We have developed a methacholine provocation method, which detects bronchial responsiveness in 80% of healthy subjects. The method enables us to detect differences in bronchial responsiveness within the normal range. With this method FEV1 and Gaw had similar sensitivity in detecting small differences in bronchial responsiveness. Differences, between protocols when using doubling or fourfold concentration steps emphasize the importance to strictly adhere to a predefined protocol. Deep inhalation associated with the FEV1 manoeuvre decreases bronchial tone induced by methacholine for up to 6 minutes, which emphasizes the importance of exact timing between successive FEV1 measurements in bronchial provocation tests. There is a substantial overlap in bronchial responsiveness between healthy and asthmatic subjects and a deep inhalation at the end of the methacholine test challenge could not discriminate between asthmatic and non-asthmatic subjects.

Inhalation of dust in a swine confinement building causes an intense airway inflammatory reaction with an extensive migration of inflammatory cells, predominantly neutrophils, into the upper and lower airways. Bronchial responsiveness to methacholine increased by about 3 doubling concentration steps and was normalized one week after exposure. However, exposure to dust in a swine confinement building did not yield increased bronchial responsiveness to eucapnic hyperventilation with dry air which is often observed in asthmatic subjects. Exhaled NO was approximately doubled five hours after exposure and in the present study we found no relationship between exhaled NO levels and bronchial responsiveness in healthy subjects.

Protection with half-mask inhibited the dust induced increase of exhaled NO whereas the increase in bronchial responsiveness was influenced only to a minor extent. These findings, do not support the hypothesis that the increased bronchial responsiveness following organic dust exposure is directly caused by the inflammation. Instead, a possible direct effect on the smooth muscle and swelling of the airway mucosa and increased secretions due to the general inflammatory reaction probably leads to airway narrowing enhancing the post-exposure bronchial response to methacholine.

**Key words**: Airway responsiveness, methacholine provocation, nitric oxide, eucapnic hyperventilation, organic dust.
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<td>Bronchoalveolar lavage</td>
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<td>BHR</td>
<td>Bronchial hyperresponsiveness</td>
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<td>BR</td>
<td>Bronchial responsiveness</td>
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<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
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<td>Cys-LT</td>
<td>Cysteinyl-leukotrienes (LTC4, LTD4, LTE4)</td>
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<td>DI</td>
<td>Deep inhalation</td>
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<td>DRS</td>
<td>Dose-response slope</td>
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<td>FEV1</td>
<td>Forced expiratory volume in one second</td>
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<td>Forced vital capacity</td>
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<td>Airway conductance</td>
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<td>PC20_{FEV1}</td>
<td>Provocative concentration of methacholine causing a 20% decline in FEV1</td>
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<td>Cumulative provocative dose of methacholine causing a 20% decline in FEV1</td>
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<td>PG</td>
<td>Prostaglandin</td>
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<td>Raw</td>
<td>Airway resistance</td>
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<td>RV</td>
<td>Residual volume</td>
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<td>TGV</td>
<td>Thoracic gas volume</td>
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<td>TLC</td>
<td>Total lung capacity</td>
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<td>TNF</td>
<td>Tumour necrosis factor</td>
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<td>VC</td>
<td>Vital capacity</td>
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1. Introduction

1.1 Bronchial responsiveness

1.1.1 Airway responsiveness to constrictors

Airway responsiveness describes the tendency of the airway to constrict to non-allergic stimuli, such as spasmogenic chemical mediators or physical stimuli [1, 2]. The response to bronchoconstrictors, differs between asthmatic and healthy subjects in many aspects. The concentration or the dose of a bronchoconstrictor that yields a certain degree of bronchoconstriction (PC or PD) is lower and the decrease in lung function as a function of a given agonist is steeper in asthmatic than in healthy subjects. This indicates altered sensitivity to the constrictor in asthmatic subjects and it does not imply a basic difference in the sensitivity of the bronchial smooth muscle itself [3].

![Dose-response curves](image)

**Figure 1.** Dose-response curves for normal subjects and subjects with mild and moderate asthma (modified from reference [3]).
In healthy subjects there is a plateau of the concentration/dose response to histamine and methacholine (defined as a change in FEV1 <5% over two or more of the final doses) [4, 5]. Thus, bronchoconstrictors seem to induce a more severe airway narrowing in asthmatic than in healthy subjects. Considering these findings, one of the characteristic features of asthma seems to be the absence of factors that counteract airway narrowing as a response to bronchoconstrictors. If there is a lack of counterregulatory bronchodilating mechanisms it may also explain that tolerance to repeated methacholine challenge as has been observed in healthy but not in asthmatic subjects [6].

A deep inhalation relaxes the airway smooth muscles in healthy subjects, which leads to an attenuated effect to a bronchoconstrictor. This bronchodilating effect of a deep inhalation is smaller and may even reverse and become a bronchoconstrictive effect in asthmatic subjects [7, 8]. It has been suggested that lung inflation is responsible for the maintenance of airway patency in healthy humans and that the loss of this function in asthma plays a major role as an underlying abnormality leading to the phenomenon of bronchial hyperresponsiveness [9].

Pre-challenge pulmonary function is positively correlated to the bronchial responsiveness [10, 11] and bronchial responsiveness is an important risk factor for the development of asthma [12, 13]. A longitudinal study has confirmed that bronchial responsiveness declines from childhood to adolescence, paralleling the increase in lung function and that airway calibre is a strong and independent determinant of bronchial responsiveness [14]. In other studies it has been demonstrated that increased BR was strongly and independent associated with FEV1 and FEV1/FVC [13, 15, 16]. Other factors such as airway symptoms, atopy, gender and smoking have been suggested to
correlate to bronchial responsiveness [15-19]. Increased airway responsiveness is also observed in allergic rhinitis [20], smokers (COPD) [21][22], cystic fibrosis [23] and in subjects exposed to different environmental agents [24, 25]. There is an overlap with regard to the bronchial responsiveness between asthmatic and non-asthmatic subjects and some asymptomatic individuals show high responsiveness to direct stimuli [26].

1.1.2 Airway hyperresponsiveness

“Airway hyperresponsiveness” or “bronchial hyperresponsiveness” refers to a condition in which the airways exhibit an exaggerated response to a bronchoconstrictor [27, 28]. In Sweden the prevalence of hyperresponsiveness to methacholine in a population, when BHR was defined as a PD20 of ≤ 1.6 mg [29], was 10.6% in men and 15.0% in women [30]. Bronchial hyperresponsiveness was in that study associated with self-reported asthma and rhinoconjunctivitis.

Airway hyperresponsiveness is often acquired and inhaled allergens or chemical pulmonary sensitizers can shift the responsiveness from being normal to increase transiently or for longer periods. Genetic factors could operate by determining the initial level of responsiveness or the predisposition to acquire hyperresponsiveness following exposure to appropriate stimuli. Different mechanisms may interact to cause airway hyperresponsiveness [28, 31, 32]. One mechanism could be responsible for the observed difference in airway responsiveness between normal and asthmatic subjects [3]. Other mechanisms may be responsible for the changes in airway
hyperresponsiveness in healthy subjects and in asthmatic subjects during the course of the disease. The airways of the asthmatics seem to respond with a greater constriction to a certain stimulus than airways from healthy subjects. This altered reaction may be explained by different mechanisms. The properties of the airway wall may be changed leading to reduced counteracting forces [33] or by a narrowing of the airway lumen (mucus secretion, airway wall oedema, plasma leakage into the airways) that appears in connection with the stimulus [34]. Bronchial hyperresponsiveness seems to be related to airway inflammation but it is not clear whether the inflammatory reaction causes bronchial hyperresponsiveness or if these two findings are parallel phenomena. There are a number of inflammatory cells and mediators that have the potential to participate in the development of increased airway responsiveness. Thus eosinophils, neutrophils [35], mast cells and alveolar macrophages [36] could be assumed to be of importance in bronchial hyperresponsiveness [37, 38]. In addition, there seems to be a relationship between the number of activated (CD25 positive) T-lymphocytes and bronchial methacholine responsiveness [39].

1.2 Provocation tests

1.2.1 History

1920-1930 Weiss et al [40] demonstrated histamine-induced bronchoconstriction in asthmatic and healthy subjects.
1940- Tiffeneau [41] measured bronchial hyperresponsiveness as a diagnostic test for asthma.

1970- Many different methods (and modifications of methods) for provocation challenges have been developed and provocation tests are induced in clinical practice:

1975 Chai [42], Orehek [43]
1977 Cockcroft [44]
1978 Juniper [45]
1981 Ryan [46]
1983 Hargreave [47], Yan [48], Eiser [49]
1986 Hendrick [50]
1988 Nieminen [51]

Table 1. Provocation stimuli

<table>
<thead>
<tr>
<th>Direct stimuli</th>
<th>Indirect stimuli</th>
<th>Both direct and indirect stimuli</th>
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<tr>
<td>Histamine</td>
<td>Cold/Dry air</td>
<td>Leukotrienes</td>
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<tr>
<td>Methacholine</td>
<td>Exercise</td>
<td>Tachykinins</td>
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<tr>
<td>Charbachol</td>
<td>Adenosine</td>
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<tr>
<td>Acetylcholine</td>
<td>Mannitol</td>
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<tr>
<td>Prostaglandin D2</td>
<td>Hypertonic saline</td>
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<td>PAF</td>
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<td></td>
<td>Propranol</td>
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<td>SO2</td>
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1.2.2 Direct stimuli

Direct stimuli have a direct effect on the smooth muscles in the airways. Bronchoconstricting agents with a direct effect include chemical mediators of which histamine [52] and methacholine [47] have been the most useful, to assess bronchial responsiveness. Examples of other direct stimuli are shown in table 1.

Histamine and methacholine provocation test

Challenge with histamine and methacholine has a high sensitivity and reproducibility but low specificity [45, 53]. Technically it is easy to perform and the equipment costs are low.

Histamine is an inflammatory mediator producing airway obstruction through neurogenic mechanisms, direct effect on the histamine receptors and by influencing the microvascular permeability [54-56]. Methacholine acts specifically on muscarinic cholinergic receptors on airway smooth muscle. Because of side effects methacholine provocation enables us to administrate higher doses than what is possible with histamine.

Two types of methacholine protocol are commonly used, the continuous, tidal breathing method and the metered dosimeter method. With tidal breathing methods aerosols are inhaled during quiet tidal breathing at spontaneous or fixed breathing frequency for a fixed time [44, 45]. This method has been modified with drying of the nebulisate to reduce pharyngeal deposition [43, 49]. With dosimeter methods aerosols are administrated during inspiration only and mostly during deep inspirations to total lung capacity (TLC) [42, 46, 50, 51].
Response to methacholine challenge is often measured as change of forced expiratory volume in one second (FEV1) or airway resistance/conductance. Various methods of lung function assessment highlight different aspects of lung mechanics. For example, airway resistance is suggested to demonstrate changes in central airways while FEV1 measures both central and peripheral airway obstruction [49]. FEV1 measurement includes a deep inhalation and is highly reproducible but less sensitive. Airway conductance is a more sensitive index of airway calibre.

1.2.3 Indirect stimuli
Indirect stimuli probably stimulate the release of a number of endogenous mediators and activate neurogenic mechanisms, indirectly leading to smooth muscle contraction and possibly to inflammatory changes in the airway wall. Exercise, cold and dry air, have been frequently used but several new stimuli have been introduced in research (table 1). Indirect challenges may be more specific for asthma and may better reflect current status in asthmatic subjects than direct challenge [56-59].

Exercise/Cold/dry air provocation
It is well known that exercise and cold air induce bronchoconstriction in asthmatic subjects. It was concluded that the stimulus for exercise-induced bronchoconstriction was loss of either heat or water or both from the airways during exercise [60]. There are, however, results indicating that the water content of the air rather than the temperature is of importance for the development of bronchoconstriction [61, 62, 38]. Two theories have been suggested for the mechanism by which cold/dry air induces
bronchoconstriction [63]. Breathing of dry air leads to desiccation and thereby increased osmolarity of the airway lining fluid leading to mediator release from a wide variety of cells [64, 65]. The contraction of the bronchial smooth muscle response to these mediators causes the airway narrowing. Another theory is based on the assumption that inhalation of cold air leads to vascular effects resulting in vascular engorgement and mucosal swelling which lead to bronchial obstruction [66]. The thermal effects are proposed to cause a reactive hyperaemia of the bronchial microvasculature and oedema of the airway wall. Hyperventilation with cold and dry air distinguishes equally well between subjects with and without exercise-induced bronchospasm and bronchospasm induced of cold air while exercise test is less sensitive [53].

1.2.4 Allergens and Occupational sensitizers

The airway response to allergen and chemical sentitizers is more complex than the response to other bronchoconstrictive stimuli. Those “specific” bronchial provocations are used for the confirmation of allergic and occupational asthma. In connection with this type of provocation challenges there is a need for extensive safety requirements. Repeated allergen exposures induce increased bronchial reactivity and eosinophilic inflammation [67, 68]. There is an early response, predominantly caused by airway smooth muscle contraction, 10-20 minutes after inhalation and a late response probably caused by both airway smooth muscle contraction and inflammation occurring after 3 hours to several days [69]. The increase in bronchial responsiveness after allergen inhalation occurs in association with the allergen-induced late asthmatic response [70]. Controlled standardized allergen inhalations tests [71] may be helpful in confirming
allergic asthmatic responses where immunotherapy is planned and in research for investigating the pathophysiology of asthma and studying new pharmacological agents.

Specific bronchial provocations tests with occupational agents may cause early, late or dual reactions, characterized by airflow obstruction and airway hyperresponsiveness [72]. The sensitivity, specificity, and reproducibility of the agents have to be assessed before they can be recommended for general clinical use. Therefore, agents with minor previous experience should be considered as research procedures. To avoid false-negative results the method of administration and exposure should aim at reproducing the occupational situation as far as possible [73].

1.3 Deep inhalation

Deep inhalations may protect against bronchoconstrictive stimuli and induce bronchodilation. However, the effect of deep inhalations is different depending on whether the inhalations are performed before or after a methacholine challenge [74]. Deep inhalations prevent bronchoconstriction in healthy subjects [9, 75] but not in asthmatic subjects [76]. The bronchodilating effect of deep inhalations is smaller in asthmatic than in healthy subjects and it may even cause bronchoconstriction in asthmatics [8, 77, 78]. Mechanical breaking of actinomyosin bridges in airway smooth muscles and relaxant factors from airway tissue may underlie the bronchodilating effect. Stretching of airway smooth muscles and activation of neural and hormonal pathways,
where nitric oxide could be the major transmitter has been suggested as the mechanism behind the bronchoprotective effect of deep inhalations [79].

1.4 Nitric oxide

1.4.1 History

1980 Furgott and Zawadaski [80] discovered that the vasodilator response to acetylcholine was mediated by the release of a vasodilator substance from the vascular endothelium.

1987 Ignarro et al [81] showed that this endothelium-dependent relaxing factor was nitric oxide.

1991 Gustafsson et al. [82] measured endogenously produced nitric oxide in exhaled air.

1.4.2 Exhaled NO

The synthesis of NO is mediated by NO synthase (NOS) and three isoforms of the enzymes are known, two constitutive forms cNOS (endothelial NOS and neuronal NOS) and one inducible form iNOS [83]. The constitutive isoform produce NO in low concentrations while the inducible NOS produce NO in high concentrations. All three forms of NOS have been shown to be present in the airways [84, 85]. In the airways nitric oxide can be produced by the epithelium, bronchial and tracheal smooth muscles and macrophages. Endothelial and neural cells also contribute to airway NO production.

Nitric oxide has vasodilating and bronchodilating effects and antibacterial properties [86, 87]. Exhaled NO has been suggested to be a non-invasive marker of airway inflammation [88-91], and has also been used in therapy for
1.5 Exposure

1.5.1 Occupational exposure

Four work-related disorders, characterized by airflow obstruction and airway hyperresponsiveness, have been described. These conditions are occupational asthma, byssinosis, reactive airway dysfunction syndrome (RADS) [111], and variable obstruction caused by irritant substances.

Occupational asthma is defined as asthma that is either induced or exacerbated by exposure to a specific agent found at work. Occupational asthma contributes 5-10% of the total asthma prevalence [112]. The prevalence of occupational asthma varies depending on the nature of the industrial agent, concentration of exposure and the working condition.

Bronchial hyperresponsiveness and asthma can be induced or exacerbated at work by several agents, which can be separated into high and low molecular weight agents. Various agents containing proteins of high molecular weight cause occupational asthma, usually by IgE dependent mechanisms (enzymes and allergens). Of low molecular agents chemical irritants are found and the most important in this group are polyisocyanates and their oligomers [113,
Other examples of exposure inducing occupational asthma and other airway disorders are inhalation of metallic dust [115], wood dust [25, 116], textile dust [117] and other chemical substances.

1.5.2 Exposure to farming environment

Exposure in a swine confinement building may cause increased body temperature and symptoms such as fatigue, chills and headache. The exposure also induces an acute inflammation in the lower and upper airways in healthy subjects [118, 119]. The cellular reaction is dominated of neutrophils [120], but alveolar macrophages and activated T cells [121] are also increased in bronchoalveolar lavage (BAL) fluid. There is an increase of pro-inflammatory cytokines such as tumour necrosis factor (TNF), interleukin (IL)-1α, IL-1β, IL-6 and IL-8 in bronchoalveolar and nasal lavage fluid [122-124]. TNF-α and IL-6 are also increased in peripheral blood a few hours after exposure [125, 126]. The inflammatory reaction displays several similarities to pathological conditions observed in chronic bronchitis and COPD [98, 99].

In addition there is a marked increase in bronchial responsiveness to methacholine seven hours after start of the exposure [24]. There are findings indicating that mast cell derived mediators like cysteinyl leuktrienes and prostaglandin D₂ may be involved in the increased bronchial responsiveness [127].

Exposure in pig farming may lead to prolonged inflammation of the airways and risk for development of chronic airflow limitation [128-132]. Possible agents in the dust responsible for the reaction could be feed components (grain dust, glucans) and microorganisms or microbial products in the dust such as endotoxin (lipopolysaccharids, LPS) mainly from Gram-negative bacteria and
peptidoglycans mainly from Gram-positive bacteria [124]. Inhalation of irritant gases such as ammonia, hydrogen sulphide, methane and carbon dioxide may also induce acute health effects including irritation and inflammation of the airways and mucous oedema formation.
2. Aims of the Thesis

- To develop a methacholine provocation method, which discriminates between differences in bronchial responsiveness within a non-asthmatic population. Furthermore, to develop a methacholine provocation method that should be able to detect small changes in bronchial responsiveness assessed in healthy subjects following exposure to occupational agents.

- To study if deep inhalations after a methacholine provocation test discriminate, between asthmatic and non-asthmatic subjects.

- To study airway responsiveness and inflammation and the relationship between them in subjects exposed to organic (swine) dust.
3. Subjects and Methods

All studies were approved by the local ethics committees and all subjects gave their informed consent to participate in the studies. The methods are briefly summarised below and detailed descriptions of methods are provided in papers I-VI.

3.1 Subjects

3.1.1 Subjects in methacholine standardization studies
In study I, 410 non-atopic men with no history of asthma or other lung or allergic diseases performed methacholine challenge with doubling (n=101, mean age 36 (range 20-65)) or fourfold (n=309, mean age 36 (range 19-78)) increases of the concentrations. In addition 30 healthy volunteers (12 women), mean age 37 (range 21-61) performed methacholine test with both fourfold and doubling concentrations on different days.

In study II, 1038 methacholine provocation tests were performed in 769 subjects from different studies on different working populations or healthy reference populations. FEV1 and Gaw were studied as the outcome of the tests. Subjects with atopy (n=72, 32% men, mean age 24 (17-41)) and without atopy (n=207, 46% men, mean age 31 (18-76)), sawyers (n=54, men, mean age 37 (20-60)) and referents (n=32, men, mean age 43 (23-63)) were compared. In addition 37 healthy volunteers (49% men, mean age 37 (20-60), were exposed in a swine confinement building and bronchial challenges before and after exposure were compared. Reproducibility of the methacholine provocation method was studied in 41 subjects.
In study III, 16 healthy non-smoking subjects (5 men), mean age 40 (range 25-49) participated. Some subjects participated in two or more parts of the study. Group A, 10 healthy subjects performed a methacholine provocation test on two occasions, with 6 or 3 minutes interval between the concentration steps. Group B, 6 healthy subjects inhaled an identical methacholine dose ten times on different days. On five occasions deep inhalations (DI) were performed before the inhalation and on the other five days no DI manoeuvres were performed. The first FEV1 measurement was performed 2, 3, 4, 6 or 10 min after the start of methacholine inhalations. Group C, 9 healthy subjects inhaled the same methacholine dose on three different days. In one test DI manoeuvres were not performed. In the second test DI were performed before and in the third test immediately after methacholine inhalation. Group D, 9 healthy subjects inhaled a methacholine dose in two tests, one without DI manoeuvres and one with DI before the test. In this group airway resistance, thoracic gas volume (TGV) and FEV1 were measured. In group A, B and C FEV1 was measured as the outcome. The methacholine dose was identical in all tests in a given subject and corresponded roughly to the PD20FEV1 of the subject.

In study IV, 164 subjects (81 men), mean age 41 years (range 22-61), who participated in a population study performed methacholine challenge with deep inhalations immediately after the end of the provocation test. Subjects consisted of 33 asthmatic and 131 non-asthmatic subjects. Among the non-asthmatic subjects 48 had a positive skin prick test and 83 had a negative skin prick test.
3.1.2 Subjects exposed in a swine confinement building

In study V, 22 healthy, non-smoking subjects (7 men), mean age 24 years (range 21-39) without atopy or asthma were exposed in a swine confinement building. Eleven subjects performed methacholine provocation test and 11 subjects performed bronchoprovocation with eucapnic hyperventilation of dry air, one or two weeks before and 7 hours after exposure.

In study VI, 33 healthy, non-smoking, non-atopic subjects (14 men), mean age 26 years (range 20-47) with no symptoms of asthma, were exposed in the swine house. Eleven subjects were wearing a half mask and 22 subjects had no mask during exposure. All subjects performed methacholine challenge and exhaled NO was measured one week before and 7 hours after exposure.

3.2 Methods

3.2.1 Lung function (study I-VI)

Vital capacity (VC) and FEV1 were measured with a wedge spirometer (Vitalogaph®, Buckingham, UK) and VC, TLC, RV and Raw with a body plethysmograph (Eric Jaeger GmbH & Co, Würsberg, Germany) according to the American Thoracic Society criteria [133]. Local reference values were used [134, 135].

3.2.2 Airway conductance (study III)

Airway resistance was measured during tidal volume breathing with the flow-interrupter technique with a mobile equipment (AW-screen, Eric Jaeger
GmbH & Co, Würsberg, Germany) [136-139]. Conductance was calculated as 1/ \( R_{\text{int}} \).

3.2.3 Methacholine provocation test (study I-VI)

Bronchial challenge with methacholine was performed either with a jet nebulizer (MA2, Astra Meditec, Gothenburg, Sweden) or a Sidestream jet nebulizer (Medic-Aid, Pagham, UK). To standardize our method using two different nebulizers a factor to correct for the methacholine “salt output” (the amount of the methacholine solution that actually leaves the liquid phase as droplets and not by evaporation) was used, 0.93 for the MA2 nebulizer and 0.75 for the Sidestream nebulizer, respectively [140]. The nebulizers were attached to a drying device with a volume of 3.4 L. Dry air was used to produce the aerosol and served as additional air and the systems produced a constant total airflow of 24 L\,min^{-1} (0.4 L\,s^{-1}). Droplets of the aerosol evaporate (during approximately 8.5 sec) within the drying device implicating an output of a methacholine aerosol consisting of small particles. The mass median aerodynamic diameter (MMAD) of dried nebulisate was 1.7 (±1.3)\,\mu m (geometric mean) [141]. Inhalation time (2 s) and thereby volume (0.8 L) and number of breaths (15/min) were controlled using a metronome and inhalation flow was controlled by the use of a back valve at the outlet of the tube.

Pre-challenge spirometry was measured 5 min before the methacholine test. Methacholine inhalations were performed during 1 min (i.e. totally 0.5 min of inhalation and 0.5 min of exhalation). Two different methacholine provocations protocol were used. Subjects inhaled the diluent followed by 0.5 mg\,ml^{-1} methacholine. In the doubling protocol the subsequent concentrations
were 1, 2, 4, 8, 16, 32 (and in some cases 64) mg ml\(^{-1}\) and in the fourfold protocol 2, 8 and 32 mg ml\(^{-1}\), respectively.

When the methacholine test was performed in subjects with severe asthma lower concentrations (0.03, 0.06, 0.125 and 0.25 mg ml\(^{-1}\)) were used. Airway resistance was measured between 2 and 3 min and FEV\(_1\) was measured 4 min after the start of an inhalation. Exactly 6 min lasted between the start of the inhalations of two successive methacholine concentrations. By avoiding deep inhalations the apparent sensitivity to methacholine is increased [142]. Therefore, deep inhalations are minimized during the challenge and only one FEV\(_1\) measurement is allowed at each dose-step.

**Figure 2.** The Sidestream tower nebulizer system.
The result was expressed as the concentration (PC20) and the cumulated dose (PD20) methacholine causing 20% decrease in FEV1 or Gaw. In addition the dose-response slope (DRS) of FEV1 or Gaw was calculated with linear regression [143-145].

\[
PC20 = \text{antilog} \left[ \log C1 + \frac{(\log C2 - \log C1)(20-R1)}{R2 - R1} \right] \\
C1 = \text{second last concentration} \\
C2 = \text{last concentration} \\
R1 = \% \text{ fall FEV1 after } C1 \\
R2 = \% \text{ fall FEV1 after } C2
\]

3.2.4 Dry air provocation test (study V)
Eucapnic hyperventilation test was performed with dry room temperature air with 5% CO2 (Ailos Asthma test®) and with a target ventilation of 35 x FEV1 x 0.75 for 4 minutes. Pre-challenge spirometry was measured and was then performed between 1 and 20 minutes after the hyperventilation. The maximal FEV1 decrease from baseline value in was recorded [146-149].

3.2.5 Exhaled nitric oxide (study IV)
Exhaled NO was determined during single-breath exhalations, with a flow of about 100 mL s\(^{-1}\) [150]. Before the measurement mouthwash with sodium bicarbonate (10%) was performed during one minute to reduce contamination from the oral cavity [151]. Inspiration of NO-free air via a mouthpiece of total lung capacity was followed immediately by full exhalation through the mouthpiece into the apparatus. Nitric oxide was measured with chemiluminescence after reaction with ozone (Aerocrine NO-system, type EBA:1, Aerocrine AB, Stockholm, Sweden) [110, 152]. The mean value of three measurements was considered for evaluation.
3.2.6 Symptoms (study VI-V)
Symptoms like headache, chills, mental fatigue, muscle pain and malaise were recorded using a visual analogue scale (VAS) ranging from 0 - 100 mm (0 = no symptoms, 100 = severe symptoms).

3.2.7 Questionnaire (study VI)
The questionnaire was based on Obstructive Lung Disease in Northern Sweden (OLIN) questionnaire [153] and was filled in together with trained interviewers.

3.2.8 Type-1-allergy (study II-III and VI)
Atopy was assessed by skin prick tests with a standard panel of the 10 most common allergens in Sweden (Pharmacia, Uppsala, Sweden, and ALK, Copenhagen, Denmark) or with Phadiatope® (Pharmacia, Uppsala, Sweden).

3.2.9 Exposure (study VI-V)
IOM filter cassettes (25mm) (SKC Ltd, Dorset, U.K.) and IOM suction pumps (SKC Ltd, Dorset, U.K.) were used to monitor inhalable dust levels and the cassettes were equipped with polycarbonate filter (pore size 5 μm) (Millipore, Sweden). Inhalable dust was measured by a standard weighing procedure. Endotoxin analysis was made with a chromogen version of Limulus amebocyte lysate assay (QCL-1000, Endotoxin, BioWhittaker, Walkersville, USA).
3.3 Statistics

Results of lung function measurements are presented as mean value (standard deviation or 95% confidence interval) and methacholine and NO results as median value (25th and 75th percentiles). Wilcoxon signed rank test was used for paired comparisons (pre- and post-exposure) and differences between groups were assessed by Mann-Whitney U test and $\chi^2$-test (bronchial responsiveness and NO). Student’s t-test was used for analysis of lung function and symptoms. Differences (repeated measurements) in lung function and NO were compared with ANOVA. Correlations were estimated by Spearman Rank correlation test. A p-value <0.05 was considered significant.
4. Results and Discussion

4.1 Standardization of methacholine method

4.1.1 Methacholine concentrations

Our standard methacholine provocation test was modified by using fourfold increasing concentrations instead of doubling steps in order to reduce the duration of the test from maximally 60 to 30 minutes. Both protocols were used in several studies of changes in bronchial responsiveness induced by occupational irritants or sentitizers. In study I, the doubling and the fourfold protocol were compared in two groups, 410 non-atopic men (fourfold protocol n=309, doubling protocol n=101) and in a randomised trial with the same 30 subjects. When plotting FEV1 changes over log concentration or cumulated dose of methacholine, a curvilinear relation was found. In non-atopic men, when using the long protocol (doubling) PC20 was 10.9 (3.9 - 45.5) mg/mL, PD20 was 4.2 (1.6 - 20.0) mg and DRS was 3.80 (1.10 - 9.77) % mg\(^{-1}\). When using the short protocol (fourfold) PC20 was >32 (8.7 - >32) mg/mL, PD20 was >13.7 (2.6 - >13.7) mg and DRS was 2.14 (1.26 - 6.46) % mg\(^{-1}\). Thus, using doubling concentrations, PC20, PD20 and DRS could be defined in a higher proportion of healthy subjects than a protocol using fourfold concentration increase. These results were confirmed in the randomised trial with long protocol (PC20 = 4.1 (2.2 - 17.6) mg/mL, PD20 = 2.1 (0.9 - 7.3) mg, DRS = 6.17 (2.29 – 2.04) % mg\(^{-1}\)) and short protocol (PC20 = 19.5 (8.1 - >32) mg/mL, PD20 = 5.3 (2.3 - >13.7) mg, DRS = 3.09 (2.19 - 6.76) % mg\(^{-1}\)).

Depending on the fact that there are fewer inhaled concentrations to reach the same dose in the fourfold protocol than in the doubling it is thus possible that
the smooth muscles are less contracted before the dose when FEV1 decreases ≥ 20%.

Figure 3. Bronchial responsiveness measured with doubling (n=101) and fourfold (n=309) increases of the methacholine concentration in healthy, non-atopic men.

4.1.2 Lung function measurement
Forced expiratory volume in one second (FEV1) and airway resistance has been used as the outcome of provocation challenges. Airway resistance is mainly influenced by changes in central airway tone, while FEV1 is influenced by both central and peripheral airway obstruction [49]. In study II, we demonstrated that the ability to detect differences in bronchial responsiveness between groups, or to detect changes in bronchial responsiveness following exposure was approximately the same for FEV1 and Gaw (1/Rint). When the difference was small, e.g. in wood workers, FEV1 but not Gaw discriminated between exposed subjects and referents. In study II Gaw was measured using the flow-interruption technique which is carried out during spontaneous
breathing with no requirement of deep inhalations. Our methacholine provocation protocol allows only one deep breath every 6 minutes. FEV1 and Gaw had similar sensitivity to detect small differences in bronchial responsiveness in healthy subjects. The result may differ in asthmatic subject and when using other methods [154].

**Table 2.** Difference in methacholine dose-response slopes (DRS), % mg⁻¹. Mean (95% CI).

<table>
<thead>
<tr>
<th></th>
<th>logDRSFEV1</th>
<th>p</th>
<th>logDRSGaw</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Atopy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>absent</td>
<td>0.69 (0.60 – 0.77)</td>
<td>0.003</td>
<td>0.99 (0.92–1.06)</td>
<td>0.006</td>
</tr>
<tr>
<td>present</td>
<td>0.94 (0.78 – 1.10)</td>
<td></td>
<td>1.18 (1.05 – 1.31)</td>
<td></td>
</tr>
<tr>
<td>2) Saw dust</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>referents</td>
<td>0.30 (0.08– 0.52)</td>
<td>0.02</td>
<td>0.75 (0.60 – 0.91)</td>
<td>0.70</td>
</tr>
<tr>
<td>sawyers</td>
<td>0.52 (0.40 – 0.64)</td>
<td></td>
<td>0.78 (0.68 – 0.88)</td>
<td></td>
</tr>
<tr>
<td>3) Swine dust</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before</td>
<td>0.33 (0.07– 0.59)</td>
<td>0.001</td>
<td>0.86 (0.66 – 1.05)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>after</td>
<td>1.21 (0.94– 1.49)</td>
<td></td>
<td>1.65 (1.44– 1.86)</td>
<td></td>
</tr>
</tbody>
</table>

There was a significant correlation between DRSFEV1 and DRSGaw (r=0.86, p<0.0001) and between PD20FEV1 and DRSFEV1 (r=0.92, p<0.001). A 20% change in FEV1 corresponded to a 39% change in Gaw. This is in good agreement with others who expressed PDGaw as the dose of methacholine causing a 35% or 40% decrease in Gaw [155]. However, the relationship between DRSFEV1 and DRSGaw appeared slightly curvilinear.
In subjects with less sensitive airways the change in Gaw was proportionally larger, whereas in subjects with more sensitive airways, a change in FEV1 induced by methacholine corresponded to a small or no change in Gaw. A possible explanation to this finding may be that the bronchial response to methacholine in subjects with low sensitivity mainly includes larger airways (reflected as Gaw-change) whereas in subjects who are more responsive to methacholine smaller airways probably are involved (FEV1–changes).

![Graph of DRS FEV1 vs Gaw](image)

**Figure 4.** Relation between DRS for FEV1 and Gaw.
Regression equation: $\log(\text{DRS}_{\text{FEV1}}) = 1.07 \log(\text{DRS}_{\text{Gaw}}) - 0.36$, $r^2=0.75$.
Broken line indicate the identity line.
4.1.3 Effect of deep inhalations

The provoked bronchoconstriction is often evaluated by measuring the forced expiratory volume in one second (FEV1), which requires a maximal inspiration prior to the forced expiration [144, 156]. In study III, an attempt to shorten the time interval between the dose-steps in a stepwise methacholine provocation test from 6 to 3 minutes resulted in an attenuated response to methacholine. When the interval between FEV1 measurements was 5 minutes, FEV1 decreased by 2.6 (1.9-5.2)% per mg of inhaled methacholine and when the interval was 2.5 minutes FEV1 decreased by 1.7 (0.8-2.3)% mg⁻¹, (p<0.01). FEV1 was measured twice with 1 minute (long protocol) and 0.5 minute (short protocol) intervals. The second FEV1 measurement was higher in both protocols, but the difference between the first and second FEV1 only reached significance in the short protocol. FEV1 (at the highest dose of methacholine) were 87 (6.5)% and 90 (5.2)% of basal level, respectively (p<0.05).

We anticipated that the difference between the long and short protocol was that the FEV1 manoeuvre at one dose step influenced the FEV1 measurement at the subsequent dose step. A deep inspiration prior to a provocation challenge is known to prevent changes in FEV1 in asthmatic subjects, but the effect is related to the severity of the asthma and may even have a constrictor effect [157]. When a first post-methacholine FEV1 was measured 2, 3, 4, 6, and 10 minutes after a single-dose methacholine inhalation, significant differences were demonstrated between tests with and without DI, up to 6 but not 10 minutes after the start of the inhalation.

If the DI was performed immediately after the methacholine inhalation (instead of immediately before) the attenuation of the methacholine-induced
bronchoconstriction was less marked. When DI was performed before methacholine inhalation, the first FEV1 was 84 (9)% of basal value. When DI was performed after methacholine inhalation FEV1 (of basal value) was 78 (10)% and if no DI was performed FEV1 was 71 (13)%, respectively. There was difference between pre- and post-methacholine DI (p=0.05). If airway resistance was measured there was no significant difference. Raw increased from 0.13 (0.06) to 0.56 (0.36) kPa L\(^{-1}\) s after inhalation of methacholine if pre-methacholine DI was not performed, and from 0.15 (0.11) to 0.52 (0.28) kPa L\(^{-1}\) s if DI was performed. The effect of a DI may be larger in peripheral airways than in central airways, thus, a DI causes a greater increase in FEV1 than in total airway resistance [8].

Recent studies have shown that there probably are different effects of deep inspirations on constricted and non-constricted airways. Deep inspirations may have a bronchoprotective or a bronchodilating effect depending whether the DI is performed before or after the bronchoconstriction [74, 79] and the effect may appear differently in healthy and asthmatic subjects. In study IV, we demonstrated that bronchodilation induced by DI in methacholine-induced bronchoconstricted airway was similar in healthy non-asthmatic and asthmatic subjects. In 33 asthmatics, the maximal decrease in FEV1 after methacholine challenge was reduced by 15 (12)% compared to 13 (11)% in 131 healthy non-asthmatic subjects.

Mechanical breaking of actinomyosin bridges in airway smooth muscles and relaxant factors from airway tissue are suggested as mechanisms behind the bronchodilating effect of DI on constricted airways and stretching of the airway smooth muscles make the muscles more resistant to bronchoconstrictor stimuli.
4.1.4 Reproducibility

The reproducibility of the methacholine provocation method using FEV1 and Raw-measurements, the latter by the mobile flow-interruption technique, was estimated in study II. The reproducibility of PC20FEV1, PD20FEV1, DRSFEV1 and DRSGaw values were similar. The 95% confidence interval for the ratio of the first and second measurement was 0.42 to 2.26 indicating that a second measurement was roughly ± one doubling concentration of the first. The reproducibility was calculated according to accepted statistical method by Bland & Altman [158] and was found to be similar, to the reproducibility found by others [45, 144, 159].

4.1.5 Airway responsiveness levels

Because of the supply of different provocation methods it is difficult to establish a normal range of challenge test results since, the levels of PD/PC and slope levels are depending on the method. Distinguishing normal from abnormal airway responsiveness is also complicated by the overlap of responsiveness between healthy and hyperresponsive subjects. Therefore, most attempts to define a cut off level between normal and increased bronchial responsiveness have failed [11]. The levels of bronchial responsiveness to methacholine using our method are given in table 3.

Table 3. Bronchial response to methacholine challenge, doubling protocol (PC20FEV1, PD20FEV1, DRSFEV1). Median (25\textsuperscript{th} –75\textsuperscript{th} percentiles).
<table>
<thead>
<tr>
<th></th>
<th>PC20&lt;sub&gt;FEV1&lt;/sub&gt; mg/mL</th>
<th>PD20&lt;sub&gt;FEV1&lt;/sub&gt; mg</th>
<th>DRS&lt;sub&gt;FEV1&lt;/sub&gt; % mg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy, non-atopic Subjects (n=83)</td>
<td>6.3 (2.3–25.7)</td>
<td>2.6 (0.9–11.7)</td>
<td>7.5 (1.5–21.6)</td>
</tr>
<tr>
<td>Healthy, non-atopic Men (n=101)</td>
<td>10.9 (3.9–45.5)</td>
<td>4.2 (1.6–20.0)</td>
<td>3.8 (1.1–9.8)</td>
</tr>
<tr>
<td>Atopic, non-asthmatic subjects (n=48)</td>
<td>2.7 (1.5–10.2)</td>
<td>1.1 (0.6–4.5)</td>
<td>16.0 (4.4–34.8)</td>
</tr>
<tr>
<td>Subjects with mild asthma (n=33)</td>
<td>0.8 (0.5–1.1)</td>
<td>0.2 (0.1–0.4)</td>
<td>78.7 (54.1–181)</td>
</tr>
<tr>
<td>Swine dust exposed (n=11) (n=22)</td>
<td>0.7 (0.5–0.9)</td>
<td>0.2 (0.1–0.3)</td>
<td>100 (65.5–207)</td>
</tr>
<tr>
<td></td>
<td>0.7 (0.5–1.3)</td>
<td>0.2 (0.1–0.4)</td>
<td>91.8 (38.8–210)</td>
</tr>
</tbody>
</table>

4.2 Healthy subjects exposed in a swine confinement building

4.2.1 Lung function and bronchial responsiveness to methacholine

Results from the bronchial provocation test with methacholine in study V and VI (and study II) demonstrate a marked increase in bronchial responsiveness, in healthy non-asthmatic subjects following exposure in a swine confinement building (table 3). This increases in bronchial responsiveness by about 3 doubling concentration steps occurred together with symptoms like shivering, headache, malaise and increased body temperature. Following exposure in a swine house there was a slight decrease in VC (2-3%) and FEV₁ (6%) compared with pre-exposure values. Airway resistance increased significantly (p<0.05) in study V but there was no significant change in RV and TLC. Increased responsiveness to methacholine in normal naive subjects has been reported in previous studies [24, 119, 127, 160]. Increased responsiveness to histamine has also been reported using the same exposure model [161].
In study V, the duration of an increased bronchial responsiveness and lung function impairment was measured and tests were performed 1, 2, and 4 weeks after exposure. After one week bronchial responsiveness had returned to normal (PD20FEV1 = 1.38 (0.75 – 7.20) mg before and 1.40 (0.65 – 6.21) mg one week after exposure). Cormier et al. also found that subjects who were exposed to swine building environment and had post-exposure increase in bronchial responsiveness returned to pre-exposure levels 2-3 weeks later [119].

In 11 subjects the increase in bronchial responsiveness was influenced only to a minor extent by the use of a half mask during exposure (study VI). PD20FEV1 decreased from 3.3 (2.6 – 14.1) mg to 1.0 (0.39 – 2.8) mg (1.5 doubling concentration steps) when using half–mask and from 1.8 (0.77 – 9.0) mg to 0.2 (0.10 – 0.45) mg (2.7 doubling concentration steps) in those without mask (p =0.07). In a previous study Dosman et al. demonstrated that also intervention with N-95 particulate respirator reduced, but not abolished increase in the bronchial responsiveness after exposure in a swine confinement facility [162]. This suggests that components other than particles may be responsible for the increased bronchial responsiveness.
4.2.2 Bronchial responsiveness to eucapnic hyperventilation

The response following swine dust exposure includes mast cell activation [127]. Others have claimed that mast cells are involved in bronchoconstriction induced by dry air in asthmatic subjects [163, 164] and we hypothesized that subjects exposed to organic dust may react similarly i.e. with increased bronchial responsiveness to inhaled dry air following exposure to organic dust. However, we could not demonstrate any difference in bronchial responsiveness to eucapnic hyperventilation in healthy non-asthmatic subjects seven hours after exposure in a swine confinement building (study V). Dry air induced a FEV1 change of –4.3 (-7.2 to –1.8)% before and -4.8 (-6.7 - 1.6)% after exposure (p=0.72). In two subjects of eleven performing provocation with dry air FEV1 was reduced more than 15% following exposure. These two
subjects had no history of asthma and they denied current respiratory infection, still an ongoing viral infection may be the explanation. We have previously found that urinary excretion of $9\alpha,11\beta$-PGF$_2$ (a mast cell derived PGD$_2$ –metabolite) and LTE$_4$ increases following exposure in swine house but this was not accompanied by increased bronchial responsiveness to dry air [127].

![Dry air-induced FEV1 decrease (%)](image)

**Figure 6.** Bronchial response to eucapnic hyperventilation of dry air following exposure to dust in a swine confinement building (n=11). Mean values and 95% confidence intervals for the FEV$_1$- decrease, before and after (7 h, 1, 2 and 4 weeks) exposure to dust.

4.2.3 *Exhaled nitric oxide*
Exhaled nitric oxide increased in 33 healthy volunteers from 7.5 (5.7 – 13.7) ppb before exposure to 13.4 (10.5 – 17.5) ppb 5 hours after exposure in a swine confinement building (p=0.0001), (study VI). Kölbeck et al. [161] were not able to demonstrate any effect on exhaled NO while Kirsten et al. [165] reported elevated levels of nasal nitric oxide production after swine house dust exposure.

![Graph showing exhaled NO levels](image)

**Figure 7.** Exhaled NO levels before and after methacholine challenge and inhalation of β2-agonist before and after exposure in a swine house. Mean values and 95% confidence interval. **P=0.003 compared with baseline, # p=0.01 compared with the value obtained 7h after exposure.

In 11 subjects NO was measured repeatedly (fig 7). There was no difference if exhaled NO was measured 5 or 7 hours after exposure. The methacholine challenge induced a small decrease in NO and a possible explanation may be that repeated spirometries and deep breaths reduce the concentration of
exhaled NO [108, 109, 166, 167]. Inhalation of a β2-agonist (salbutamol) did not further alter the levels of exhaled NO which is in accordance with findings in asthmatic subjects where regular use of short- and long-acting β2-agonists did not alter the levels of exhaled NO [168].

In another 11 subjects who were wearing half-mask during exposure, the increase in exhaled NO was abolished. Exhaled NO was 8.3 (6.1 – 14.1) ppb before and 8.6 (6.6 – 14.6) ppb after exposure (p=0.48). There was a significant difference between subjects using a half-mask and subjects without mask (p=0.006). Particles and microbial agents which stimulate the NO production are filtered by the half-mask and therefore, the exhaled NO-levels are kept unchanged after exposure.

A high correlation between exhaled NO and airway hyperresponsiveness has been demonstrated in both mild and severe asthma [169-171]. In the present study we found no relationship between exhaled NO and bronchial responsiveness in healthy subjects following exposure in a swine confinement building (r=0.08, p=0.44).
5. General Discussion

5.1 Methacholine provocation method
The methacholine provocation method used in the present studies was primarily designed to be used in a normal population exposed to environmental factors that may cause small changes in airway responsiveness. The test should be able to discriminate between different levels of airway responsiveness within a non-asthmatic population and has enough accuracy to detect changes in bronchial responsiveness over time. The standard methacholine methods have been designed to detect asthma in clinical practice and there has been no need to define a concentration or dose causing a certain change in lung function in subjects with normal bronchial responsiveness [42, 44]. Thus, the main purpose with our method was not to design a method capable to separate non-asthmatic subjects from subjects with asthma. The method was influenced by the method described by Orehek and Eiser [49] and the device was designed to ensure delivery of a known amount of agent to the lower airways. For this purpose inspiratory flow was held low and constant and the nebulisate was dried. With this method it has previously been demonstrated that 87% (6,7) of the inhaled dried nebulisate reaches the lower airways compared to 54% (10.7) without the use of the drying device [141]. The duration of a standard bronchial provocation test with doubling doses of methacholine is usually 30 to 60 minutes. With the aim to create a more rapid test we found several factors that influence the result of a methacholine provocation test.

We shortened the time interval between the dose steps and modified the test by using fourfold concentration steps instead of doubling steps. Using
doubling concentrations, PC20 and PD20 could be defined in a higher proportion of healthy subjects than a protocol using fourfold dose increases (study I). Although, methacholine has a “small” cumulative effect [45] the difference between the two protocols was not depending on whether the bronchial effect is regarded as cumulative or non-cumulative. However, the difference between the groups was smaller if the provocative dose rather than the concentration was used, suggesting that cumulative dose is a better estimate than the concentration. Fewer inhaled doses to reach the same dose, and therefore less contracted smooth muscles before the dose when FEV1 decrease ≥20% in the short protocol than in the long protocol may explain the differences. However, the magnitude of such effect is unclear and other unknown factors may also contribute to the observed effect. Because of smaller differences in methacholine concentration between the different dose steps, the long protocol (doubling concentrations) is the most preferable method to assess changes and differences in bronchial responsiveness.

We also found that a shortened time interval between the dose steps (from 5 to 2.5 min between FEV1 measurement) in the provocation test reduced the decrease in FEV1 caused by methacholine (study III). One explanation could be insufficient time for full effect of nebulized methacholine at the time point of the first post-methacholine FEV1 in the short protocol. However, the most plausible explanation is that FEV1 measured at one dose step may influence the FEV1 measured at the subsequent dose step. We have demonstrated that the duration of the effect when DI was performed before inhalation of methacholine remained 6 but not 10 minutes. Ten minutes after the inhalation of methacholine, when the effect of methacholine is still maximal [172] the level of the first post-challenge FEV1 was almost identical when a deep
inhalation had or had not been performed. A deep pre-methacholine inhalation did not significantly influence airway resistance. The data suggest that the effect of DI may be larger in peripheral than in central airways and probably depends on a larger distending force in the peripheral airways. A partial relaxation of constricted peripheral airways smooth muscles due to a DI may thus cause a greater increase in forced expiratory flow than in total airway resistance.

Kapsali et al demonstrated that the response to methacholine in asthmatic subjects was not affected by deep inspirations [76]. It is suggested that stretching of the airway, which should occur as a result of lung inflation, activates a biochemical process that changes the resting state of the airway smooth muscle or operates in functional antagonism against bronchoconstrictive stimuli. This airway stretch may activate neural pathways or release of bronchodilators such as nitric oxide in non-asthmatic subjects. This effect of lung inflation may become defective in asthma [9, 79]. Nadel and Tierney [7] reported that in normal subjects deep inspiration transiently reduced and even abolished histamine-induced increase in bronchomotor tone. In asthmatic subjects the bronchodilating effect of deep inspirations after induced bronchoconstriction has been shown to be smaller than in non-asthmatics and deep inhalations may even potentiate bronchoconstriction [8]. In our study deep inhalations attenuated the methacholine-induced bronchoconstriction similarly in asthmatic and non-asthmatic subjects (study IV). The result is in accordance with those of Scichilone and co-worker [79], although they demonstrated that the bronchodilator effect in healthy subjects was of a somewhat greater order of magnitude than in asthmatics. It has been claimed that mechanical breaking of actinomysin bridges in airway smooth
muscles and relaxant factors from airway tissue may be responsible for the bronchodilatation.

With this methacholine provocation method, PC20 was defined in almost 80% of the healthy. The results from study IV, demonstrated difference in bronchial responsiveness between subjects, healthy non-atopic, atopic non-asthmatic and asthmatic subjects. The results also demonstrated that the bronchial responsiveness to methacholine in many healthy subjects is identical to what is found in mild asthma and deep inhalations at the end of the methacholine challenge could not discriminate between asthmatic and non-asthmatic subjects.

Both FEV1 and Gaw have been used to measure the airway response of methacholine provocation tests. It has been suggested that airway conductance is more sensitive to reveal methacholine-induced bronchoconstriction than FEV1. Orehek et al showed that FEV1 is less sensitive than airway resistance as an outcome measure of induced bronchoconstriction in asthmatic subjects because of the influence of the preceding or following FEV1 [157]. The measurement of airway conductance does not require deep inhalation and the effect of deep inhalations seems to have a less influence on Gaw [173] [142].

Using a methacholine provocation protocol, which only allows one deep breath every 6 minutes we found that FEV1 and Gaw had similar sensitivity in detecting small differences in bronchial responsiveness (study III). Airway resistance is mainly influenced by changes in central airway tone, while FEV1 is influenced by both central and peripheral airway obstruction [49]. Thus, if the difference was very small with no affection on central airways as in sawmill workers [25], FEV1 but not Gaw would discriminate between exposed subjects and referents.
5.2 Bronchial responsiveness following exposure in swine confinement building

The mechanisms underlying the increased bronchial responsiveness in healthy subjects exposed in a swine confinement building are still unclear. The activation of mast cells (urinary increase in 9α,11β-PGF₂α) made us to hypothesize that cys-LTs might contribute to the increased bronchial responsiveness [174]. The participation of leukotrienes in the inflammatory reaction, induced by exposure in a swine house, has been demonstrated by increased excretion of urinary LTE₄, and increased levels of LTB₄ and LTE₄ in nasal lavage fluid [127]. Since it has been claimed that mast cells are involved in the bronchoconstriction induced by dry air in asthmatic subjects [163, 164] we hypothesized that subjects exposed to organic dust also react with increased bronchial responsiveness following inhalation of dry air. However, bronchial responsiveness in subjects exposed in swine confinement building did not increase after provocation to dry air like that seen in asthmatics. Thus, airway inflammation of different “profiles” is related to airway hyperresponsiveness with different features. There was no alteration in the bronchial response when pharmacological intervention with, the 5-lipoxygenase inhibitor (Zileuton) was used during 5 days prior to the exposure [175]. In another study, sodium cromoglycate inhibited dust induced airway cytokine (TNF and IL-6) release and neutrophil influx without influencing the increase in bronchial responsiveness to methacholine [160]. The almost total inhibition of TNF (assessed in BAL fluid) without any alteration of increase in bronchial responsiveness may speak against a central role of this cytokine in the
development of increased bronchial responsiveness following exposure to dust.

Pig farmers had increased concentrations of neutrophils in BAL fluid compared to a reference group with normal bronchial responsiveness [176]. However, we have demonstrated that farmers have less airway reaction to acute exposure in a swine confinement facility than non-farmers, suggesting possible adaptation mechanisms in chronically exposed swine farmers [177]. Reduced FEV1 and airway symptoms (wheezing and shortness of breath), have been reported to be associated with bronchial hyperreactivity in farmers [178]. In another study Pedersen et al [179] demonstrated that pig farmers with normal lung function had macroscopic signs of bronchial inflammation and an increased number of neutrophils in BAL.

The inflammatory reaction (increased concentration of both neutrophils and IL-8) following dust exposure in healthy subjects showed similarities with the airway inflammation observed in chronic bronchitis and COPD [128, 180]. Although the 75-fold increase in neutrophilic granulocytes was the most notable finding in the inflammatory response there were also a significant increase in eosinophilic granulocytes after exposure in a swine house [120]. It can thus not be excluded that the eosinophils could be of importance for the development of increased bronchial responsiveness [181]. It has been claimed that eosinophilic granulocytes are important for bronchial hyperresponsiveness in asthma [39, 182] and that eosinophils together with macrophages are producers of mediators with bronchoconstricted properties [183, 184].

In study VI, the increased levels of exhaled NO may indicate an induction of bronchoalveolar NO-synthase induced by exposure, which in turn induces
production of pro-inflammatory cytokines. Swine house dust includes exposure to LPS and the inflammatory response following this exposure comprehends increased concentrations of TNF-α in serum and in BAL [118, 123] and both LPS and TNF-α could induce iNOS expression. The increased levels of NO could reflect a normal host-defence response to bacteria in the inhaled organic dust. We were not able to demonstrate any correlation between the increase in exhaled NO and bronchial responsiveness, which indicate that these two outcome measures reflex different aspects of the airway inflammation induced by exposure to the farming environment. It has been shown that airway hyperresponsiveness correlates with exhaled NO in atopic but not in non-atopic asthmatics [185] and that there is a correlation between exhaled NO and sputum eosinophils [171].

We established that the airway inflammation following organic dust exposure is not associated with a bronchoconstrictor response to hyperventilation with dry air. This was contradictory to our hypotheses, that mast cell activation, demonstrated with increased excretion of urinary 9α,11β-PGF₂ and LTE₄, contribute to the development of increased bronchial responsiveness. Thus, airway inflammation of different “profiles” is related to airway hyperresponsiveness with different features. Hyperventilation induced bronchoconstriction seems to be a feature exclusive for asthma.

In our studies, no correlation was observed between bronchial reactivity and the inflammatory response measured as change in concentration of both inflammatory cells and mediators (cytokines, leukotrienes and PGD₂) after swine dust exposure. Hence these results do not support the hypothesis that the increased bronchial responsiveness following organic dust exposure is directly caused by mediator release.
Figure 8. Possible mechanisms behind increased bronchial responsiveness following exposure in a swine confinement building.

The airway resistance (R) is proportional to the radius of the airway lumen (r) demonstrated by the formula $R = \frac{1}{r^4}$ [186]. The reduction of the airway lumen diameter caused by swelling and secretions of the airway mucosa induced by the airway inflammatory reaction leads to a more pronounced bronchial obstruction after exposure. Thus, a healthy normal subject would display a higher tolerance to airway narrowing prior exposure in a swine farm, than what is demonstrated in the same person following exposure. Even a moderate increase in wall-thickness in the bronchioles, caused by oedema, cellular infiltration and hyperaemia, can result in increased response to
bronchoconstrictive agents in asthmatic and probably also in patients with chronic airway obstruction [34, 187].

Wearing a half-mask during exposure, filters the particles and bacterial components, and abolished completely the increase of exhaled NO-levels following exposure whereas the influence of the mask on the increase of bronchial responsiveness was minor. Also the increase in inflammatory cells in blood (neutrophils, monocytes and eosinophils) and mediators in NAL (IL-6, IL-8) were dramatically decreased in subjects wearing respiratory protection device during exposure in the swine house [188]. This finding does not support the hypothesis that the increased bronchial responsiveness following organic dust exposure is directly caused by the inflammation.

The increased bronchial responsiveness, which occurred despite the use of a respiratory protection device, may suggest a contribution of gases in the development of increased bronchial reactivity. Gases like ammonia and hydrogen sulphide are present in the farm environment but do seldom exceed hygienic threshold limits. However, in a recently performed study where 12 healthy subjects were exposed to ammonia in an exposure-chamber the exposure did not influence lung function, bronchial responsiveness or exhaled NO [189]. This was in accordance with other studies in pig farmers [180] and other workers [190] exposed to low levels of airborne ammonia, where no associations between bronchial responsiveness or lung function and ammonia were described.
6. Conclusions

The results emphasise the importance of strict adherence to a given protocol when performing methacholine provocation tests. The reasons for this are:

- Avoiding deep inhalations increase the apparent bronchial sensitivity to methacholine. A deep inhalation before methacholine inhalation influences, the outcome of the following FEV1 measurement but does not affect the following Raw-measurement to the same extent. Only one, forced expiration is allowed at each dose step.
- The time interval between inhalation of methacholine and the successive FEV1 measurements must be constant.
- It is important to use the same increasing dose steps when comparing doubling and fourfold dose step increases. The doubling protocol is preferable when assessing changes and differences in bronchial responsiveness.
- Use own reference values (using the identical method) is of importance when comparing studies and when choosing a cut-off value for bronchial hyperresponsiveness.

With this methacholine provocation method:

- PD20 was defined in almost 80% of all subjects when using the doubling interval protocol.
- FEV1 and Gaw had similar sensitivity in detecting differences in bronchial responsiveness in healthy non-asthmatic subjects.
However, at small differences, FEV1 was better to discriminate between groups. The reproducibility was similar (within one doubling concentration), for FEV1 and Gaw.

- A 20% change in FEV1, correspond to a 39% change in Gaw.
- There is a high correlation between PD20FEV1 and DRSFEV1 ($r^2=0.85$).
- The method discriminates (although with some overlap) between non-asthmatic subjects without negative skin prick test, non-asthmatic subjects with positive skin prick test and asthmatic subjects.
- Deep inhalations after the provocation may not discriminate between asthmatic and non-asthmatic subjects. Deep inhalations attenuate methacholine-induced bronchoconstriction in both groups.

Exposure in a swine confinement building leads to:

- Symptoms and increased body temperature.
- A slight lung function, (VC and FEV1) impairment (3 and 6%, respectively).
- Increase in bronchial responsiveness to methacholine (approximately 3 concentration steps) 7 hours after exposure. After 1 week responsiveness was back to pre-exposure levels.
- Unaltered bronchial responsiveness to eucapnic hyperventilation with dry air.
- Increase in exhaled NO (approximately doubled). There was no correlation between increase in NO and PD20FEV1. Use of $\beta_2$-agonists does not affect the NO values.
• Using half-mask abolished the increase in exhaled NO but influenced the increased bronchial responsiveness to methacholine only to a minor extent.

These results indicate that the features of increased bronchial responsiveness after exposure to organic dust are different from the features of bronchial hyperresponsiveness in asthma. Gas components rather than particles in the air may be responsible for the increased bronchial responsiveness. Airway inflammation is not the only mechanism behind the hyperresponsiveness following exposure in a swine confinement building (fig.8).
7. Sammanfattning

Under utarbetande av ett provokationstest med metakolin som gör det möjligt att mäta skillnader i bronkiell reaktivitet hos friska personer, som exponeras för olika agens i miljön fann vi flera faktorer, som påverkade utfallet av testet. Olika koncentrationer av metakolin (0.5, 1, 2, 4, 8, 16 och 32 mg/ml) inhaleras normalt under ett test tills FEV1 sjunkit med 20% jämfört med värdet före provokationen. Om koncentrationsökningen utfördes med fyr-dubbla koncentrationssteg med syfte att göra testet snabbare, framstod försökspersonerna mindre känslig i luftvägarna. Även när tiden mellan metakolininhalationerna förkortades (från 6 till 3 minuter) minskade känsligheten. Orsaken till dessa skillnader kan vara påverkan av olika antal djupa andetag som görs i samband med FEV1 mätningar, eftersom djupa andetag påverkar sammandragna luftvägar hos friska personer längre än 6 minuter efter inhalation av metakolin. Djupa andetag utförda efter ett metakolintest har även förmågan att dilatera sammandragna luftrör hos både friska försökspersoner och personer med lätt astma.

Mätning av konduktansen (Gaw) utförs utan djupa andetag och det har antytt att Gaw skulle vara ett känsligare mått att mäta förändringen av bronkiell reaktivitet under metakolinprovokationen. Därför undersöktes de båda variablernas (Gaw och FEV1) möjlighet att mäta den bronkiella reaktiviteten. Mellan atopiker och icke atopiker, sågverksarbetare och kontroller och hos personer före och efter exponering i svinhus diskriminerade Gaw och FEV1 lika bra. Slutsatsen av dessa resultat är att det är viktigt att man använder samma metod och protokoll, för att kunna göra jämförelser mellan olika grupper och studier vid mätning av bronkiell reaktivitet.
Friska försökspersoner utvecklar en kraftig luftvägsinflammation, karakteriserad av en invandring av inflammatoriska celler, huvudsakligen neutrofiler, efter tre timmars exponering i svinhus. Den bronkiella reaktiviteten för metakolin (direkt stimuli), mätt med vår metod, ökade med ungefär 3 koncentration dubblingsteg, men hade återgått till basalvärdet 1 vecka efter exponeringen.

Eftersom mastceller har hävdats vara involverade i bronkkonstriktionen orsakad av hyperventilation med torrluft (indirekt stimuli) hos astmatiker och att förhöjda halter av PGD₂, en mastcells produkt, har uppmätts i urin efter exponering, antog vi att personer exponerade i svinhus skulle reagera på samma sätt. Vi kunde inte påvisa någon känslighet mot torr luft hos de svinhusexponerade vilket tyder på att den ökade bronkiella reaktiviteten hos dessa är annorlunda än den hos astmatiker.

Personer exponerade i svinhus dubblerade sin NO-halt (inflammations markör), 5 timmar efter exponering, men vi fann inget samband mellan den ökade bronkiella reaktiviteten och NO. Andningsskydd under exponeringen tog bort ökningen av utandat NO men en ökning av den bronkiella reaktiviteten kvarstod, om än i mindre grad. Avsaknad av samband mellan uppmätta inflammatoriska mediatorer som bla. NO och ökad bronkiell reaktivitet tyder på att den ökade luftvägsreaktiviteten inte direkt orsakades av inflammationen. Tänkbara orsaker kan istället vara en ökad svullnad och sekretion i luftvägarnas slemhinna leder till luftvägsförträngning, vilket i sin tur resulterar i en kraftigare luftvägsobstruktion vid inhalation av samma dos metakolin efter exponering och en eventuell direkt påverkan på den glatta muskeln.
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