Airway responsiveness to methacholine: effects of deep inhalations and airway inflammation

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Airway responsiveness to methacholine: effects of deep inhalations and airway inflammation. J. Appl. Physiol. 87(2): 567–573, 1999.—We determined the dose-response curves to inhaled methacholine (MCh) in 16 asthmatic and 8 healthy subjects with prohibition of deep inhalations (DIs) and with 5 DIs taken after each MCh dose. Flow was measured on partial expiratory flow-volume curves at an absolute lung volume (plethysmographically determined) equal to 25% of control vital capacity (FVC). Airway inflammation was assessed in asthmatic subjects by analysis of induced sputum. Even when DIs were prohibited, the dose of MCh causing a 50% decrease in forced expiratory flow at 25% of control FVC (PD25MCh) was lower in asthmatic than in healthy subjects (P < 0.0001). In healthy but not in asthmatic subjects, repeated DIs significantly decreased the maximum response to MCh [from 90 ± 4 to 62 ± 8 (SD) % of control, P < 0.001], increased PD50MCh (P < 0.005), without affecting the dose causing 50% of maximal response. In asthmatic subjects, neither PD50MCh when DIs were prohibited nor changes in PD50MCh induced by DIs were significantly correlated with inflammatory cell numbers or percentages in sputum. We conclude that 1) even when DIs are prohibited, the responsiveness to MCh is greater in asthmatic than in healthy subjects; 2) repeated DIs reduce airway responsiveness in healthy but not in asthmatic subjects; and 3) neither airway hyperresponsiveness nor the inability of DIs to relax constricted airways in asthmatic subjects is related to the presence of inflammatory cells in the airways.

bronchial asthma; healthy; partial flow-volume curve; induced sputum

AIRWAY HYPERRESPONSIVENESS has long been recognized as a hallmark of bronchial asthma (24a, 25), but the underlying mechanisms are still largely obscure. Although a causal relationship between airway inflammation and airway hyperresponsiveness can be reasonably postulated, clear evidence for this hypothesis is lacking (6). In 1981, Fish et al. (8) first submitted that airway hyperresponsiveness may reflect inability to dilate constricted airways rather than increased response to stimuli. This hypothesis has recently received support by Skloot et al. (31), who found similar methacholine (MCh) dose-response curves in asthmatic and normal subjects when deep inhalations (DIs) were strictly prohibited. A serious limitation of this study is that the index of bronchoconstriction used, i.e., the time constant of partial forced expiration, may be highly sensitive to changes in lung volume (4). Burns and Gibson (4) measured the instantaneous flow at constant absolute lung volume on partial expiratory flow-volume (PEFV) curves and found greater responses to MCh in asthmatic than in normal subjects. A limitation of this study is that a DI was required after each PEFV curve to determine absolute lung volume. Neither Skloot et al. (31) nor Burns and Gibson (4) examined the possibility that the response to MCh in asthmatic subjects was related to the degree of airway inflammation. Therefore, differences in subject characteristics between the two studies cannot be excluded.

In the present study, we measured instantaneous flow on PEFV curves in which absolute lung volume was determined by body plethysmography, thus making it possible to construct MCh dose-response curves without DIs, independent of changes in lung volume. The purposes of the study were the following: 1) to give a definite answer on whether prohibition of DIs makes the MCh dose-response curve in healthy subjects similar to that in asthmatic subjects, 2) to investigate to what extent imposition of repeated DIs affects the MCh dose-response curve in healthy and asthmatic subjects, and 3) to investigate whether asthmatic airway inflammation influences the MCh dose-response curve or the effects of DIs on it.

METHODS

Subjects. Twenty-four subjects (8 who were healthy and 16 with mild asthma) participated in the study after giving informed consent. The two groups were similar in terms of age and baseline lung function, whereas the degree of airway responsiveness to MCh, determined on a previous occasion by a standard protocol (5), was greater in the asthmatic than in the normal subjects (Table 1). None of the subjects was a smoker or had suffered from viral infections of the upper respiratory tract in the previous month before the study. The asthmatic subjects were in stable condition and were taking only short-acting (β2)-agonists on demand, which were avoided for 12 h before the study. Subjects allergic to pollens were studied out of season. All subjects, who were well trained in performing respiratory maneuvers, attended the laboratory on two occasions to undergo two different MCh challenges (see below). The asthmatic subjects attended the laboratory on a third occasion for sputum induction. In all subjects the study was completed within 5 days.

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Table 1. Demographics and functional characteristics

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Asthmatic subjects

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FEV₁, forced expiratory volume in 1 s; Pred, predicted; FVC, forced vital capacity. *From Ref. 29; PD₂₀MCh, dose of methacholine causing a 20% decrease in FEV₁ with a standard challenge protocol; M, male; F, female; +, positive; −, negative. †Geometric. ‡Significantly different from healthy subjects, P < 0.0001.

Lung function measurements. A Vmax 6200 Autobus System (SensorMedics, Yorba Linda, CA) was used for all lung function measurements. Thoracic gas volume (TGV) was measured by whole body plethysmography while the subject panted slightly below 1 Hz against a closed shutter. Residual volume (RV) was obtained by subtracting from TGV the maximum volume that could be exhaled after the opening of the shutter. Total lung capacity (TLC) was obtained by adding redistilled water to dry, powdered MCh chloride (Laboratorio Farmaceutico Lofarma, Milan, Italy). Aerosols were delivered during quiet tidal breathing by an SM-1 Rosenthal Breath-Activated Dosimeter (SensorMedics) driven by compressed air (30 psi) with 1-s actuations. The aerosol output at the mouth was 10 µl/actuation. Twenty inhalations of saline were given as a control, and then MCh was given in doubly increasing doses up to a maximum of 4.8 mg (noncumulative), unless the subject asked for interruption at lower doses. The dose increments were obtained by using two concentrations of MCh (1 and 10 mg/ml) and increasing the number of breaths from 2 to 48.

Two challenge procedures were used at random. In protocol 1, the subject continued to breathe quietly after MCh inhalations until lung function measurements were started. In protocol 2, the subject took five consecutive DIs from FRC to TLC immediately after each MCh dose before entering the plethysmograph for lung function measurements. In both protocols, PE芙V curves were obtained ~2 min after each MCh dosing.

Dose-response curves. The changes in Vp₂₅ were plotted against MCh doses. The maximal response of each individual dose-response curve was determined by fourth-order polynomial fitting. Bronchoconstriction was considered "unlimited" if no plateau of response was identified by inspection of the fitted curve below a 90% decrease in Vp₂₅. The doses causing a 50% decrease in Vp₂₅ from control (PD₅₀MCh) and 50% of maximal response (ED₅₀MCh) were calculated by linear interpolation.

Sputum induction and analysis. After inhalation of a buffered (200 µg by metered dose inhaler), ultrasonically nebulized (Devilbiess 65, Devilbiess, Somerset, PA) hypertonic (4.5%) saline was inhaled for periods of 1, 2, 4, 8, and 16 min. After each inhalation period the subject was asked to rinse his or her mouth with water and cough to produce sputum. Within 2 h from collection, the whole sputum sample was examined by inverted microscopy, and portions were selected to minimize salivary contamination. Dithiothreitol (Sputolyxin, Calbiochem, San Diego, CA) diluted (1:10) in distilled water was added in a volume equal to twice the weight of the selected sputum portion. After 20 min in a shaking water bath (37°C), the sample was further diluted with PBS in a volume equal to that of sputum plus dithiothreitol and PBS. After mucus was removed by filtering through sterile gauze, the suspension was centrifuged at 1,000 g for 5 min, and supernatants were aspirated and stored (−70°C). The cell pellet was resuspended in a volume of PBS equal to that of the filtered suspension, and total cells were counted by a Burker's chamber hemocytometer. The cell suspension was then centrifuged at 450 rpm for 6 min (Shandon Cytocentrifuge, Shandon Southern Instruments, Sewickley, PA). Differential counts of 500 nucleated nonepithelial cells were made on two cytospin slides stained and stained by May Grunwald Giemsa. Cytospins with >20% squamous epithelial cells or <50% cell viability were discarded.

Statistical analysis. Anthropometric and baseline lung function data were analyzed by descriptive statistics and unpaired Student's t-test. The differences in maximal response and PD₅₀MCh between asthmatic and healthy subjects with or without DIs were tested by a mixed (between-within groups) repeated-measures ANOVA. PD₅₀MCh and ED₅₀MCh values were log transformed before statistical analysis and are presented as geometric means. Other data are presented as means ± SE. The relationships between PD₅₀MCh or its DI-induced changes and numbers (or percent-
healthy and 13 asthmatic subjects. PD 50MCh (Fig. 2) was significantly less in asthmatic than in healthy subjects (geometric mean: 21 vs. 169 µg, P < 0.0001) but not in asthmatic subjects.

Values are means ± SE. V˙p25, instantaneous flow from partial flow-volume curve at 25% of control forced vital capacity. Note that dose of MCh causing 50% of maximal response (ED50MCh) was unchanged by DIs (arrows). Note also decrease in maximal response was not significantly different in the two groups (99 ± 1% in asthmatic vs. 90 ± 4% in healthy subjects, P > 0.6). Unlimited decrease in V˙p25 was observed in 6 healthy and 13 asthmatic subjects. PD50MCh (Fig. 2) was significantly less in asthmatic than in healthy subjects (geometric mean: 21 vs. 169 µg, P < 0.0001).

Effect of DIs (protocol 2 vs. protocol 1). In healthy subjects, the dose-response curves to MCh for protocol 2 were displaced to the right compared with those for protocol 1 (Fig. 1). Maximal response was significantly less (P < 0.001) for protocol 2 (62 ± 8% of control) than for protocol 1 (90 ± 4% of control). In all but one subject there was a plateau in V˙p25 for protocol 2. PD50MCh (Fig. 2) was significantly greater (P < 0.005) for protocol 2 (741 µg) than for protocol 1 (169 µg), but ED50MCh values were not significantly different (222 vs. 154 µg, P > 0.2). Baseline lung function on the two occasions was not significantly different: FEV1 was 3.95 ± 0.18 liters for protocol 1 and 4.01 ± 0.20 liters for protocol 2 (P > 0.6).

In asthmatic subjects, the dose-response curves to MCh for protocol 2 were not significantly different from those for protocol 1 (Fig. 1). Neither maximal response nor PD50MCh (Fig. 2) was significantly different between protocols (P > 0.9 for both comparisons). The decrease in V˙p25 was unlimited in 12 of the 16 subjects. Baseline lung function on the two occasions was not significantly different: FEV1 was 3.34 ± 0.18 liters for protocol 1 and 3.48 ± 0.20 liters for protocol 2 (P > 0.1).

Relationship to airway inflammation. Both total and differential cell counts of induced sputum were greatly variable in asthmatic subjects (Table 2). With DIs prohibited (protocol 1), PD50MCh was also greatly variable (from 5 to 118 µg) but did not correlate with any inflammatory cell number or percentage in sputum (Table 3). Also, a multiple regression model including all inflammatory cell numbers in sputum as independent variables was unable to explain a significant part of the variability of PD50MCh with DIs prohibited (r² = 0.29, P > 0.3). Also, changes in PD50MCh between protocol 2 and protocol 1 did not correlate with any inflammatory cell number or percentage in sputum by using either simple (Table 3) or multivariate analysis (r² = 0.14, P > 0.7). The relationships between PD50MCh or its DI-induced changes and the percentage of eosinophils in sputum (a widely used index of airway inflammation in asthma) are shown in Fig. 3.

DISCUSSION

The main findings of this study are the following: 1) even when DIs were prohibited, MCh dose-response curves in asthmatic subjects were different from those in healthy subjects; 2) multiple DIs during MCh challenge greatly reduced maximal bronchoconstriction and increased PD50MCh in healthy, but not in asthmatic, subjects, whereas ED50MCh was unchanged; and 3) in asthmatic subjects, neither PD50MCh with DIs prohibited nor changes in PD50MCh induced by DIs were correlated with the numbers of inflammatory cells in induced sputum.

Prohibition of DIs. Skloot et al. (31) recently reported similar MCh dose-response curves in healthy and asthmatic subjects when DIs were prohibited, but this finding was not confirmed by Burns and Gibson (4). The present study also showed different MCh dose-response curves in asthmatic and healthy subjects even when DIs were prohibited. Skloot et al. used the time con-
stant of partial expiration as an index of bronchoconstriction. This index is affected by changes in lung volumes (4) and may underestimate bronchoconstriction particularly in asthmatic subjects, in whom RV increases more. In the extreme case of a parallel shift of the descending limb of the flow-volume curve during bronchoconstriction, due to an increase in RV without a change in slope, the time constant of partial expiration would not change despite a large decrement in expiratory flows (Fig. 4). Burns and Gibson used an index that is independent of changes in lung volume, i.e., the forced expiratory flow at constant lung volume on PEFV curves, but a DI was required after completion of each PEFV curve to estimate absolute lung volume. They allowed 4 min between each DI and the next PEFV curve, but this time interval may not be sufficient for the effect of DI to disappear completely (20). We used the same index of bronchoconstriction as did Burns and Gibson, but DIs were avoided by having absolute lung volumes measured plethysmographically before each PEFV curve.

Table 2. Differential cell counts in sputum of asthmatic subjects

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Table 3. Simple correlation coefficients between airway responsiveness and inflammatory cells in induced sputum

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Values are Pearson’s correlation coefficients (\( n = 16 \)). \( \text{PD}_{50} \text{MCh} \), dose of methacholine causing a 50% decrease in partial flow at 25% of control forced vital capacity (VF\(_{25}\)) with deep inhalations (DIs) prohibited; \( \text{PD}_{50\text{Diff}} \text{MCh} \), difference in \( \text{PD}_{50} \text{MCh} \) induced by repeated DIs. None of these values was significant at \( P < 0.05 \).

In the study by Skloot et al. (31), partial forced expiratory maneuvers were started from spontaneous end-tidal volume, whereas in the present study they were started from double the spontaneous end-tidal volume. The somewhat greater stretching associated with doubling tidal volume could have shifted the dose-response curves of healthy but not of asthmatic subjects to the right, thus contributing to the difference between the two groups. This seems unlikely because, in four additional healthy subjects, the dose-response curves to MCh by using PEFV started from spontaneous end-tidal volume were not significantly different from the control values (Table 2).
from those started from double the spontaneous end-tidal volume (PD_{50\text{MCh}} geometric mean: 120 vs. 138 µg, P > 0.6). The V_{p_{25}} used in this study reflects events at very low lung volume, whereas the time constant used by Skloot et al. reflects the whole partial curve. In 7 healthy and 11 asthmatic subjects we could also construct dose-response curves by using flow measured at 40% of FVC. The difference in the PD_{50\text{MCh}} geometric mean between asthmatic (179 µg) and healthy (24 µg) subjects remained highly significant (p < 0.001), suggesting that the absolute lung volume at which airway caliber was assessed cannot explain the difference in results between this study and the study by Skloot et al. Finally, it is possible that even small differences in pattern of breathing during the whole challenge might affect airway responsiveness.

**Effect of DIs.** During induced bronchoconstriction, a single DI causes a transient increase in airway caliber both in normal (24) and asthmatic (3) subjects, although this effect is less in the latter (2, 3, 27, 28). This bronchodilator effect, which can be explained by the theory of relative hysteresis (11) without assuming changes in airway smooth muscle tension, declines with a time constant of 10–12 s (26). In protocol 2 of the present study, changes in airway caliber were inferred from PEFV curves obtained 45 s after the last DI to TLC. Therefore, the different results obtained for protocol 1 and protocol 2 in healthy subjects cannot be explained by the relative hysteresis theory. More likely, the reduced responsiveness in protocol 2 reflects a reduced force generation by airway smooth muscle.

Two theories have been proposed to explain why stretching may reduce the force produced by airway smooth muscle. According to Fredberg et al. (9) each time inspiration occurs the airway smooth muscle is elongated, because of the interdependence between airways and lung parenchyma, and rapid-cycling cross bridges are detached, thus decreasing the tensile force. It is possible that increasing the magnitude of stretching by DIs immediately after each MCh dose in protocol 2 may have resulted in a greater rate of cross-bridge detachment and, by inference, a decrease in tensile force and less airway narrowing compared with protocol 1. According to Gunst et al. (13–15), the force produced by airway smooth muscle depends on its length when the stimulus is applied. If the airway smooth muscle is stimulated when elongated, e.g., during DIs, and then brought to a shorter length, e.g., when normal tidal breathing is resumed, the force is less than if airway smooth muscle is stimulated at short length, e.g., during quiet tidal breathing. This difference has been explained by different configurations of the contractile apparatus inside the airway smooth muscle (14). In the present study, MCh was inhaled during quiet tidal breathing in both protocols to avoid any effect of different breathing patterns on aerosol deposition. After airway smooth muscle stretching, it takes time for the contractile apparatus to return to the short-length configuration (30). In protocol 2, airway smooth muscle was stretched for the first time after control inhalation, thus possibly causing a configurational change in the contractile apparatus that persisted when MCh was inhaled. Therefore, both of the above theories can explain the results of the present study.

In healthy subjects, repeated DIs reduced maximal response and increased PD_{50\text{MCh}} without affecting ED_{50\text{MCh}}. The maximal response to bronchoconstrictor stimuli in vivo is believed to be determined by the force-generation capacity of airway smooth muscle, the elastic loads opposing its shortening, and the thickness of airway walls (23). Stretching of lung parenchyma in healthy subjects may cause stress relaxation of its elastic elements, which would favor and not oppose...
In asthmatic subjects, the dose-response curves to MCh were unaffected by DIs. In most cases, an unlimited decrease in V\textsubscript{125} was achieved with both protocols. As complete airway closure may have occurred even with submaximal airway smooth muscle activation, ED\textsubscript{50}MCh could not be calculated, and the effects of DIs on maximal response and position of the dose-response curve cannot be separated. However, the similarity of PD\textsubscript{50}MCh in protocol 1 and protocol 2 suggests that DIs did not significantly affect the force-generation capacity of the airway smooth muscle.

There are various reasons why DIs may not affect the airway responsiveness to a constrictor agonist in asthma. First, the force of interdependence in asthma may be less than normal, because of a reduced lung elastic recoil (10, 21), or is poorly transmitted to the airway walls, because of peribronchial edema (18). Second, asthmatic airway smooth muscle may generate greater force because of hyperplasia or hypertrophy (7), thus making the pulling effect of DI less efficient. Third, parenchymal hysteresis may increase in response to inhaled MCh in asthma (2), resulting in a reduced load on airway smooth muscle during expiration from TLC. If the velocity of airway smooth muscle shortening is increased (22), then the contractile force may be fully reestablished before the next inspiration, thus making DIs ineffective in reducing airway smooth muscle force (32).

Relationship to airway inflammation. Previous studies, using standard challenge procedures and FEVs as an index of bronchoconstriction, did not provide clear evidence that airway hyperresponsiveness is related to the presence of inflammatory cells in the airways (6). The relationship between airway responsiveness and airway inflammation with DIs prohibited has not been investigated before. In the present study, no significant correlations were found between PD\textsubscript{50}MCh without DIs and inflammatory cell numbers in sputum, and the relationships between changes in PD\textsubscript{50}MCh induced by repeated DIs and inflammatory cells in sputum were also insignificant. It appears, therefore, that breathing maneuvers are not an important confounding factor in the assessment of the relationships between airway hyperresponsiveness and airway inflammation. On the other hand, the evaluation of prohibition or imposition of DIs on measurements of airway responsiveness does not seem to be affected by the presence of inflammatory cells in the airways. It is possible that changes affecting the response to MCh and the ability to dilate the airways with DIs reside deeper in the airway walls or at the interface between airways and lung parenchyma. Sputum analysis gives reproducible results (33) that fairly well agree with those of bronchoalveolar lavage (19) but only reveals inflammatory cells present in the bronchial lumen. Therefore, the effects of chronic airway inflammation in terms of airway wall remodeling cannot be evaluated.

Conclusions. This study confirms the conclusion by Burns and Gibson (4) that airway hyperresponsiveness is not just a problem of lack of dilatation with DI. In addition, it also shows that prohibition of DIs during a bronchial challenge greatly increases airway responsiveness in healthy subjects, thus making their MCh dose-response curve more similar (although not equal) to that of asthmatic subjects. These results have two practical implications. First, the breathing pattern during MCh challenge, both during aerosol inhalation and during lung function measurements, may profoundly affect measurements of airway responsiveness, thus making comparable only measurements obtained with the same protocol. Second, a protocol with repeated DIs allows a better separation between healthy and asthmatic subjects.

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