Dose-Response Relationship and Reproducibility of the Fall in Exhaled Nitric Oxide After Inhaled Beclomethasone Dipropionate Therapy in Asthma Patients

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Study objectives: The fractional concentration of exhaled nitric oxide (FENO) is a marker of asthmatic airway inflammation. We determined the dose response and the reproducibility of the FENO fall following inhaled beclomethasone dipropionate (iBDP) therapy in nonsteroid-treated asthmatic patients.

Study design: Study A: For four 1-week periods (period 1 to period 4), the following regimens were administered in sequential order to 15 nonsteroid-treated asthmatic patients: period 1, placebo; period 2, 100 μg/d of iBDP; period 3, 400 μg/D of iBDP; and period 4, 800 μg/d of iBDP. Spirometry, FENO, and provocative concentration of methacholine resulting in a 20% fall in FEV1 (PC20) were measured at each of five visits (visit 1 to visit 5). Study B: During four periods, 12 nonsteroid-treated asthmatic patients received placebo treatment for 7 days (period 1), 200 μg/d of iBDP for 14 days (period 2), washout on placebo treatment until the FENO was within 15% of baseline (period 3), and 200 μg/d of iBDP for 14 days (period 4).

Results: Study A: Mean FEV1 rose progressively from 3.10 L (visit 1) to 3.41 L (visit 5; p < 0.001). All iBDP doses caused a significant FEV1 rise compared to placebo treatment, but with no significant separation of doses using FEV1. FENO geometric mean (95% confidence limits) fell progressively from 103.5 parts per billion (ppb) (78.5 to 136.7) to 37.4 ppb (29.1 to 48.0) from visit 1 to visit 5 (p < 0.001). All doses of iBDP resulted in a significant change in FENO from placebo treatment, but with significant separation of only the 100-μg and 800-μg doses by FENO. Geometric mean (95% confidence limits) PC20 rose progressively from 0.01 mg/mL (0.00 to 0.19) to 0.48 mg/mL (0.01 to 8.1) from visit 1 to visit 5 (p = 0.002). All doses of iBDP resulted in a significant change in PC20 from baseline or placebo treatment, but with no significant separation of active iBDP doses using PC20. Study B: FENO fell from 111.56 ppb (80.3 to 155.1) to 66.3 ppb (49.2 to 89.5; p < 0.001) from period 1 to period 2, and from 110.2 ppb (79.3 to 153.1) to 61.7 ppb (42.9 to 88.8; p < 0.001) from period 3 to period 4. There were no significant differences between FENO in period 1 and period 3 (p = 0.83) or between period 2 and period 4 (p = 0.220).

Conclusions: FENO was superior to FEV1 and PC20 in separating doses of iBDP. The fall in FENO after two identical administrations of iBDP separated by placebo washout was highly reproducible.

Key words: asthma; dose response; exhaled nitric oxide; reproducibility; steroids

Abbreviations: AMP = adenosine-5’-monophosphate; CI = confidence interval; CL = confidence limit; FENO = fractional concentration of exhaled nitric oxide; iBDP = inhaled beclomethasone dipropionate; iNOS = inducible nitric oxide synthase; PC20 = provocative concentration of methacholine resulting in a 20% fall in FEV1; N O = nitric oxide; ppb = parts per billion

The fractional concentration of exhaled nitric oxide (FENO) has been proposed as a noninvasive marker of airway inflammation, a primary process in the pathogenesis of asthma. Nitric oxide (NO) synthesized by constitutive NO synthases mediates physiologic responses such as vasorelaxation, while inducible NO synthase (iNOS) is expressed in pathologic states and is involved in host defense and the inflammatory response.

Exhaled NO is increased in some subjects with asthma, falls after treatment with corticosteroids, and this effect is both rapid in onset and resolves rapidly on steroid therapy withdrawal. iNOS is increasingly expressed in the respiratory epithelium...
in asthma, in response to cytokines secreted from macrophages and lymphocytes. This expression has also been shown to be reduced after inhaled budesonide dipropionate (iBDP). Corticosteroids modulate the expression of iNOS via binding with nuclear factor κB in the cytosol and possibly by inhibiting cytokine synthesis.

Standard efficacy measures in asthma include symptom scores, home pulmonary function monitoring, rescue medication use, and pulmonary function testing including assessment of airway reactivity. These efficacy measures mainly assess airway obstruction rather than airway inflammation. Use of standard efficacy measures to determine a dose response for inhaled corticosteroids has not been easy, probably because of the variability in response between individuals, as well as lack of adequate sensitivity. The lack of a dose response has made it difficult to use standard efficacy measures to determine the optimal dose of inhaled corticosteroids.

Due to potential systemic effects, it is preferable to prescribe the minimal dose of inhaled steroids that will achieve adequate asthma control. This requires a sensitive and specific method for assessing airway inflammation. Using a standardized FENO measurement technique together with other standard outcomes, we examined the dose response of iBDP on the decrease in FENO. In a second study, we determined if the effect on FENO of repeated administration of the same dose of iBDP, with washout in between, was reproducible over a short time period.

Materials and Methods

Study A: Dose Response of the Fall in FENO after iBDP

Subjects: We recruited 15 asthmatic patients (8 male patients; age range, 17 to 40 years) with baseline FENO > 60 parts per billion (ppb; a value > 2 SD above mean values for healthy control subjects in our laboratory), in order to select subjects with increased baseline FENO. Asthma was diagnosed according to American Thoracic Society recommendations (1996). Exclusion criteria included the use of oral or inhaled corticosteroids, other anti-inflammatory agents, of long-acting β2 agonists in the 4 weeks prior to the study; smoking within the previous 3 years or > 5 pack-year smoking history; upper respiratory tract infection in the previous 4 weeks; pregnancy; and any other significant chronic medical disease. The study was approved by the Toronto Hospital Ethics Committee; subjects signed an informed consent form.

Study Design: This was a single-cohort, prospective study with five visits over 4 weeks. Each subject served as his or her own control on placebo and active medications. The baseline visit (visit 1) included history and physical examination, FENO, spirometry, and determination of the provocative concentration of methacholine resulting in a 20% fall in FEV1 (PC20) according to American Thoracic Society guidelines. For four 1-week periods (periods 1 to 4), the following were administered twice daily via metered-dose inhaler to the 15 subjects: period 1, placebo; period 2, 100 μg/d of iBDP; period 3, 400 μg/d of iBDP; and period 4, 800 μg/d of iBDP. After 1 week at each dose level, the subjects came to the laboratory for measurement of FENO, spirometry, and PC20.

NO Measurement: NO was analyzed using a rapid-response chemiluminescent NO analyzer (290 NOA; Sievers Instruments; Boulder, CO). The NO detection limit is approximately 1 ppb for gas, with a response time of 200 ms for 90% full scale. Data were collected via a computerized data collection program. Calibration was performed at each session. The analyzer was zeroed using 100% nitrogen (Praxair; Mississauga, Ontario, Canada) containing < 1 ppb NO. High-point calibration was performed with a commercial NO gas standard containing 2 ppm (Praxair). The analyzer sample line was connected just distal to the mouthpiece and sampled at a flow rate of 150 mL/min.

FENO Measurement Technique: We used a restricted breath technique, which employed exhalation via a high resistance, and positive mouth pressure to close the velum, thus excluding nasal NO. Subjects inhaled medical-grade compressed air (Praxair) that contained < 2 ppb NO and then exhaled via a high expiratory resistance while targeting a mouth pressure of 20 mm Hg. This produced an expiratory flow rate of 45 mL/s (including analyzer sampling rate). Exhalations were repeated until three plateau FENO values varied by < 5%. The mean of the three replicate FENO values was used in all analyses.

Spirometry and Methacholine Challenge: Spirometry was performed according to American Thoracic Society guidelines using a dry rolling seal spirometer (model 130; P.K. Morgan; Gillingham, Kent, UK) and an XY recorder (model 7045A; Hewlett Packard; Palo Alto, CA). Methacholine challenge was performed with a tidal breathing pattern using a hand-held nebulizer according to American Thoracic Society guidelines.

Statistics: The distributions of FENO and PC20 significantly deviated from normal, so data were transformed to log base 10 for FENO and log base 2 for PC20. A mixed model was used to determine if there were significant differences between any of the end points for all treatments. Post hoc analysis revealed that a compound symmetric covariance structure gave the best fit for FEV1 and an autoregressive structure with order 1 + random subject structure was the best fit for PC20 and FVC. Fairwise comparisons were then made among the four dose levels with results adjusted for multiple comparisons using the Tukey-Kramer method. All statistical tests were two sided and conducted at the 5% significance level.

Study B: Reproducibility of the Fall in FENO

Subjects: We recruited 12 different subjects (five men; age range, 18 to 24 years) with a baseline FENO > 60 ppb with identical exclusion criteria as in study A. The inclusion baseline FENO criteria and dose of medication were determined based on conclusions derived from study A.

Study Design: The design was a single-blinded, prospective study conducted over 8 to 12 weeks in four phases: period 1, placebo metered-dose inhaler (1 week); period 2, iBDP at 200 μg/d (2 weeks); period 3, washout of medication using placebo metered-dose inhaler (variable period); and period 4, identical to period 2. Each subject served as his or her own control during periods of placebo and active medications. All subjects underwent history and examination, FENO, spirometry, and PC20 at baseline. FENO was measured two to three times weekly in all periods. PC20 was measured at baseline to confirm the diagnosis of asthma, and was not an outcome measure in this study. Period 3 terminated when two sequential FENO measurements had returned to within 15% of the mean FENO recorded during period 1. If period 3 was > 6 weeks, subjects were withdrawn.
**FENO Measurement:** This was identical to that described in study A.

**Statistical Analysis:** Multiple measurements of outcome variables within periods were summarized by taking the average of the visits in the baseline period and the average of the last two visits in subsequent periods, for each subject. The data were summarized using arithmetic means and SEs, or geometric means for those variables with distributions that deviated from a normal distribution after log transformation. A mixed-effects model with a compound symmetric covariance structure was used to determine if there were mean differences for the four treatment periods accounting for repeated measures for subjects across periods.\(^{13}\) Contrasts within the mixed model were used to test for differences in pairs of means and to determine if the change in outcome from period 1 to period 2 was equal to the change from period 3 to period 4. All tests were two sided and conducted at the 5% level of significance. Analyses were performed using software (SAS version 6.12; SAS Institute; Cary, NC) on a personal computer (Windows NT; Microsoft; Redmond, WA).\(^{14,15}\)

**RESULTS**

**Study A: Dose-Response Study**

**Baseline Characteristics:** Fifteen subjects were recruited to the study. Geometric mean (95% confidence limit [CL]) baseline measurements were as follows: FENO, 103.5 ppb (78.5 to 136.7); PC\(_{20}\), 0.01 mg/mL (0.00 to 0.19); mean ± SD FEV\(_1\) and FVC at baseline were 3.01 ± 0.73 L (83.5 ± 16.6% predicted) and 4.49 ± 0.97 L (103.4 ± 12.2% predicted), respectively.

**The Dose Response of the Fall in FENO:** The general model showed that the test for treatment differences in FENO was highly significant, indicating that at least two of the treatment means differed (p < 0.001). The summarized results are shown in Table 1 and are depicted in Figure 1. There was no significant change between FENO at baseline and after 1 week of placebo inhaler treatment. There was a progressive fall in FENO as the dose of iBDP was increased, and all doses of iBDP were associated with a significant change in FENO from baseline and placebo values, even after correcting for multiple comparisons. FENO with 100 µg of iBDP was significantly different from 500 µg, but no significant differences were seen between the 100-µg and 400-µg doses or between the 400-µg and 800-µg doses.

**The Dose Response in High- and Low-FENO Groups:** A post hoc inspection separated subjects into those with baseline FENO of 60 to 100 ppb (n = 6) and > 100 ppb (n = 9), on the assumption that airway inflammation would be mild to moderate and moderate to severe in these two groups, respectively. Figure 1 shows the FENO responses of the entire cohort and the two subgroups. In descriptive terms, the low-FENO group showed a modest fall in FENO with 100 µg/d, but no further decline in FENO as the dose of iBDP was increased. The high-FENO group showed a progressive fall in FENO at each dose level, eventually reaching a similar level of FENO as the low-FENO group. Statistical analysis was not applied here due to small sample sizes.

**The Dose Response in PC\(_{20}\):** The general model showed that the test for treatment differences in PC\(_{20}\) was highly significant, indicating that at least two of the treatment means differed (p = 0.002). The summarized results are shown in Table 1 and are depicted in Figure 2. There was no significant change between PC\(_{20}\) at baseline and after 1 week of placebo inhaler treatment. However, all doses of iBDP were associated with a significant change in PC\(_{20}\) from baseline and placebo values even after

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*Data are presented as geometric mean (95% CL) or mean ± SD. p values at each dose level are compared to placebo.

†p = 0.008.

‡p < 0.001.

§p = 0.01.

¶p = 0.003.

†p = 0.025.

#p = 0.002.

**p = 0.004.
correcting for multiple comparisons (Table 1). No significant differences were seen for PC_{20} between 100 mg/d and 400 mg/d (p = 0.485), between 100 mg/d and 800 mg/d (p = 0.451), or between 400 mg/d and 800 mg/d (p = 0.977) of iBDP.

The Dose Response in PC_{20} According to Baseline FENO: A post hoc inspection separated subjects into those with baseline FENO of 60 to 100 ppb (n = 6) and >100 ppb (n = 9), on the assumption that airway inflammation would be mild to moderate and moderate to severe in these two groups, respectively. Figure 2 shows the PC_{20} responses of the entire cohort and the two subgroups. In descriptive terms, the PC_{20} response in the low-FENO group showed a progressive increase as the dose of iBDP rose. However, the PC_{20} response of the high-FENO group showed no change as the iBDP dose increased. Statistical analysis was not applied here due to small sample size.

The Dose Response of the Change in FEV{\textsubscript{1}}: The general model showed that the test for treatment differences in FEV{\textsubscript{1}} was highly significant, indicating that at least two of the treatment means differed (p < 0.001). The summarized results are shown in Table 1 and Figure 3. There was no significant change between FEV{\textsubscript{1}} at baseline and after 1 week of placebo inhaler treatment. However, all doses of iBDP were associated with a significant change in FEV{\textsubscript{1}} from placebo and baseline values after correcting for multiple comparisons, except for the 100-μg dose compared to baseline. No significant differences were seen for FEV{\textsubscript{1}} between any of the doses of iBDP. For FVC, there were no significant differences between any of the treatment levels, compared with baseline or placebo.

Study B: Reproducibility of the Fall in FENO

Twelve subjects were enrolled in this study. Three subjects failed to return to baseline FENO by 6 weeks in period 3 and were withdrawn from the study. The results below refer to the nine subjects who completed the study.
Changes in FENO: The fall in FENO started 2 to 3 days after the active treatment (Fig 4). For FENO, both prebronchodilator and postbronchodilator, significant mean reductions were observed from period 1 to period 2, and from period 3 to period 4. No significant differences were found in mean FENO between period 1 and period 3, confirming the adequacy of the washout period, or between period 2 and period 4, indicating a reproducible effect of iBDP. The p value for the test comparing the mean fall in FENO from period 1 to period 2 with that from period 3 to period 4 was not significant for prebronchodilator or postbronchodilator FENO (p = 0.470 and p = 0.385, respectively). This indicates that the effect on FENO of the two periods of iBDP treatment was reproducible (Fig 5). Excluding subject 4, who had particularly high FENO values, did not change any statistical outcomes.

Change in FEV₁ and FVC: FEV₁ progressively increased from period 1 to period 4; unlike FENO, FEV₁ did not fall on steroid withdrawal (Table 2). For FEV₁, both prebronchodilator and postbronchodilator, there were significant differences between the means values for period 1 and period 2 and for period 1 and period 3. No other significant differences were found between any periods for prebronchodilator or postbronchodilator FEV₁. No significant differences were found between any periods for FVC prebronchodilator or postbronchodilator, nor were there significant differences in the mean changes from period 1 to period 2 and from period 3 to period 4.

Discussion

Exhaled NO is widely regarded as a noninvasive marker of airway inflammation. In these two studies, we examined the dose response of sequential administration of increasing doses of iBDP on FENO, spirometry, and PC20, and also the reproducibility of the effect of a single dose of iBDP administered twice on FENO and spirometry. Exhaled NO, but not FEV₁ or PC20, distinguished the 100-µg dose from the 800-µg/d dose of iBDP. The effect of repeated administration of 200 µg/d of iBDP on both FENO and FEV₁ was highly reproducible.

In this study, FENO fell and PC20 rose significantly even with 100 µg/d of iBDP, a dose that is considered to be subtherapeutic. Furthermore, FENO, in contrast to FEV₁ and PC20, distinguished between the 100-µg/d and 800-µg/d doses of iBDP. Inspection of the high-baseline FENO group (Fig 1) suggests that a larger sample size of subjects with FENO > 100 ppb might have demonstrated a significant dose response for all iBDP doses studied. If a high-baseline FENO means more inflammation, then selection of a group on the basis of FENO may allow a dose response to be demonstrated, as the change in a parameter after an intervention will always be directly proportional to the initial absolute level of that parameter. For FENO, it appears that the dose-response levels out at > 800 µg/d of iBDP.

To our knowledge, there is only one published study using FENO to examine the dose response to inhaled corticosteroids. In three parallel groups, each containing from six to eight subjects, Jatakanon et al. examined the dose response of inhaled budesonide (placebo, 100 µg/d, and 400 µg/d) using FENO, methacholine, and induced sputum as outcome measures. In a further crossover study, 10 subjects were randomized to treatment with 1,600 µg of budesonide or placebo. There were significant improvements in FEV₁ following 400 µg and 1,600 µg/d of budesonide (11.3% and 6.5%, respectively;
p < 0.05) accompanied by significant reductions in eosinophil numbers in induced sputum (p < 0.05). However, levels of FENO were reduced following each budesonide dose, while PC20 rose only with 1,600 µg/d of budesonide. A plateau response of FENO was found at a dose of 400 µg/d of budesonide. Our study also found a detectable effect of even 100 µg/d of iBDP on FENO, but unlike Jatakanon et al.,16 this dose also affected PC20, and the FENO response bottomed out at 500 µg/d of beclomethasone, compared to 400 µg/d of budesonide. This difference may be related to differing potencies of these two compounds. Additionally, our study subjects were preselected for a raised baseline FENO.

PC20 significantly increased on as little as 100 µg/d of iBDP, but this parameter was unable to distinguish a significant dose response in the group as a whole. The high- and low-FENO groups show differing patterns of PC20 change, with the former showing a “flat” PC20 response while the latter showed a progressive PC20 rise (Fig 2). This can perhaps be explained on the basis of differing severity of airway inflammation between the two groups. The more inflamed group (high-baseline FENO) was more resistant to modulation of PC20 during the short period of this study. Longer treatment, however, at the higher dose level might have eventually resulted in PC20 modulation. This suggests that inflammation as assessed by FENO is unimportant in determining bronchial reactivity in the high-baseline FENO group. FEV1 also rose significantly with the 400-µg/d and 800-µg/d dose levels, but did not show a significant dose response.

Several designs for a dose-response study using inhaled steroids in asthma could have been employed, each with its own merits. The parallel group design, as used by Jatakanon et al.,16 avoids the need to wash out the effect of the medication, but requires larger numbers of subjects, and for the groups to be matched in baseline characteristics. The single-cohort design, with each dose followed by a washout period, is probably the best theoretical design, but takes longer, thus increasing dropout rates and the chance of asthma exacerbation. Additionally, FENO in some subjects will not return to baseline, as seen in the reproducibility study. The design we used in this study is short, without need for washout. However, there is the possibility of carryover effects from the previous dose level.

There are few studies examining the dose response of inhaled corticosteroids on bronchial reactivity. In a three-parallel-dose group, double-blind, placebo-controlled, randomized, crossover study, with a washout period of 3 to 8 weeks, Taylor et al.17 used adenosine-5’-monophosphate (AMP) to study the effect of 14 days of treatment with 100 µg/d, 400 µg/d, and 1,600 µg/d of the inhaled corticosteroid ciclesonide on airway responsiveness to AMP and inflammatory parameters in induced sputum. Compared with placebo, 100 µg/d, 400 µg/d, and 1,600 µg/d of ciclesonide reduced airway responsiveness to AMP by 1.6 (95% confidence interval [CI], −0.1 to 3.4; not significant), 2.0 (95% CI, 0.4 to 3.6; p < 0.05), and 3.4 (95% CI, 2.3 to 4.4; p < 0.05) doubling doses, respectively, and this was dose dependent (p = 0.039). A significant reduction in the percentage of eosinophils in induced sputum was observed after 400 µg/d and 1,600 µg of ciclesonide (p < 0.05) but was not dose dependent.

The short-term reproducibility of the depressant effect of 200 µg/d of iBDP on FENO shows that the effect is predictable and that in most subjects, cessation of inhaled steroid therapy results in return of FENO levels to baseline values within 1 week to 2 weeks. This also indicates that subjects tend to
return to a certain level of FENO. Indeed, we have observed that subjects who return for different studies tend to have similar FENO levels each time. Three subjects, however, were withdrawn from the study as FENO remained depressed for up to 6 weeks. The factors determining this prolonged effect are unknown. Perhaps these subjects were not reexposed to an environmental allergen. Of great interest is the contrast between the changes in FEV1 and FENO. FENO reacted rapidly to the administration and withdrawal of medication, whereas FEV1 improved progressively throughout the study. Thus, these two parameters are temporally discordant and may be assessing different components of the asthmatic state.

In summary, FENO was superior to both FEV1 and PC20 in establishing a dose response for 100 μg/d and 800 g/d of iBDP, but was unable to separate 100 μg/d from 400 μg/d, and 400 μg/d from 800 μg/d, perhaps related to sample size. The effect of iBDP on FENO is highly reproducible in the short term. Exhaled NO monitoring may be useful in determining the minimal effective dose of inhaled steroids, perhaps only in those subjects who have a raised baseline FENO, and could also be used to compare the potencies of anti-inflammatory medications.

REFERENCES