

SHORT REPORT

The effect of the nitric oxide synthase inhibitor, L-NMMA, on sodium metabisulphite-induced bronchoconstriction and refractoriness in asthma

A.M. Hamad*, A. Wisniewski*, S.P. Range*, T. Small**, F. Holland*, A.J. Knox*

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ABSTRACT: Refractoriness to indirect bronchoconstrictor stimuli, is a feature of asthma but the mechanism is poorly understood. This study tested the hypothesis that endogenous nitric oxide (NO) produced during a first bronchoconstrictor challenge protects against subsequent challenge and therefore has a role in the refractory process.

The effect of an NO synthase inhibitor, *N*^G-mono-methyl-L-arginine (L-NMMA), on refractoriness to sodium metabisulphite (MBS) was investigated in 20 subjects with mild asthma. On visit one, the dose of MBS which caused a 20% fall in forced expiratory volume in one second (FEV₁) (PD₂₀) was determined. On visit two, the refractory index (RI) to MBS was determined by challenging the subjects twice with their PD₂₀ of MBS, the second challenge proceeding after recovery from the first. Those showing a refractory index of ~30% (10 subjects) inhaled either L-NMMA or placebo followed 5 min later by two challenges with their PD₂₀ of MBS in a double-blind cross over study at two further visits.

The dose of L-NMMA used was shown to reduce exhaled NO for a duration sufficient to cover the second MBS challenge. However, no significant difference was found between L-NMMA and placebo in maximum fall in FEV₁% and area under the curve (AUC) during first or second MBS challenges or in RI on the two study days.

It is concluded that subjects with mild asthma show refractoriness to sodium metabisulphite, but that endogenous nitric oxide is unlikely to be involved either in the refractory process or in the response to sodium metabisulphite *per se*.

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*Depts of Respiratory Medicine, City Hospital, Hucknall Road, Nottingham and **Respiratory Medicine, Freeman Hospital, Freeman Road, Newcastle upon Tyne.

Correspondence: A.J. Knox
Respiratory Medicine Unit
City Hospital
Hucknall Road
Nottingham NG5 1PB
UK
Fax: 44 1158404771

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Refractoriness, a reduced response to a second bronchoconstrictor challenge after recovery from the first, is well recognized with several indirect bronchoconstrictor challenges [1–3]. This may be an important physiological protective mechanism. However, the mechanisms involved in refractoriness are poorly understood. It has previously been shown that inhibitory prostaglandins may be responsible for part but not all of the refractoriness to challenges such as sodium metabisulphite (MBS) and exercise [1, 3], but other factors are clearly important.

Nitric oxide (NO) is a potential candidate for a bronchoprotective role in the refractory process as endogenous NO acts as a bronchodilator and neurotransmitter of inhibitory nonadrenergic noncholinergic (iNANC) nerves [4]. This might be particularly important with MBS since it is thought to cause bronchoconstriction *via* a neural mechanism, possibly NANC, which would be expected to cause NO release. It was hypothesized that endogenous NO produced during a first indirect bronchoconstrictor challenge might protect against subsequent challenge. If endogenous NO is involved in refractoriness then pre-treatment with an inhibitor of NO production during a first challenge would prevent refractoriness induced by subsequent challenge. The authors therefore determined

whether inhaled *N*^G-mono-methyl-L-NMMA (L-NMMA) given immediately prior to an MBS challenge would prevent refractoriness in subjects with mild asthma.

Materials and methods

Subjects

Subjects with mild asthma, requiring inhaled β -agonist therapy only, were recruited from the City Hospital asthma register and screened for MBS refractoriness. Twenty subjects were screened and 11 had a refractory index (RI) of $\geq 30\%$ (defined as the difference between the area under the curve (AUC) for the first and second challenge as a percentage of the values of the first challenge); 10 of these agreed to enter the double-blind phase of the study. Six subjects were atopic (two or more positive skin prick tests to common allergens), and all of them were nonsmokers. All subjects were symptom-free at the time of the study and had a forced expiratory volume in one second (FEV₁) of $>70\%$ predicted [5]. None of the participants had suffered an exacerbation of asthma or respiratory infection within 2 weeks of the study. The study was approved by the Nottingham City Hospital Ethics Committee.

Study design

Subjects undertook two screening visits. On the first visit the provocative dose of MBS causing 20% fall in FEV₁ (PD₂₀) was determined and on the second visit the RI to MBS was determined. Those showing an RI of $\geq 30\%$ attended on two subsequent visits where they inhaled L-NMMA or placebo in a double-blind, cross over manner before having two sequential challenges with their PD₂₀ dose of MBS.

Methods

β -agonist treatment was withheld for 6 h before each visit. Visits were separated by ≥ 2 days and were performed at the same time of day. FEV₁ was measured as the highest of two successive readings, within 100 mL, on a dry wedge spirometer (Vitalograph, Buckingham, UK). The MBS PD₂₀ was measured by giving increasing doses to a cumulative maximum of 76.9 μmol , using a MEFAR breath activated dosimeter (MEFAR, Brescia, Italy) as described previously [6].

Screening visits

Visit 1. In the first part of the study, subjects underwent an initial MBS challenge to determine their PD₂₀ of MBS.

Visit 2. Subjects returned for a single dose challenge with the PD₂₀ of MBS identified at the previous visit. FEV₁ was measured before (baseline) and 2 min after inhalation of 3 puffs of normal saline (post-saline value) and at 1, 3, 5, 7, 10, 15 and 20 min after the MBS challenge. Subjects were rechallenged with the same dose of MBS immediately after recovery (FEV₁ $>95\%$ of post-saline value) and FEV₁ was measured at the same intervals for a further 20 min. Subjects who had a reduction in AUC of the changes in FEV₁ over 20 min at the second challenge of $\geq 30\%$ were chosen to proceed to the second part of the study.

Visit 3 and 4: study of the effect of inhaled N^G-mono-methyl-L-arginine on the airway response to sodium metabisulphite. After baseline measurement of FEV₁, each subject inhaled an aerosol of either L-NMMA (2 mg in 5 mL 0.9% saline) or placebo (5 mL of 0.9% saline) in a double-blind, cross over study. The drug was administered via a jet nebulizer (Pari Master, Starnberg, Germany) driven by a compressed air source (147 kPa) with an output of 0.50 g·min⁻¹. The subject inhaled the aerosolized solution at tidal breathing through a mouth piece until the nebulizer was dry. FEV₁ was measured 5 min after finishing the aerosol delivery, and the subjects inhaled 3 puffs of normal saline and the FEV₁ was measured again. The subjects were then challenged with MBS on two occasions as on visit 2.

Study of the effect of inhaled N^G-mono-methyl-L-arginine on exhaled nitric oxide levels

Exhaled NO was measured using a highly sensitive rapid chemiluminescence analyzer (LR2000, version 2.2; Logan Research, Rochester, UK) as previously described [7]. Three healthy nonsmoking control subjects without a history of asthma and/or wheeze were recruited from hospital staff and their exhaled NO levels were measured before (baseline) and 1, 15, and 60 min after inhalation of L-NMMA (2 mg in 5 mL 0.9% saline).

Analyses

Sodium metabisulphite PD₂₀ was calculated by linear interpolation of the log dose-response curve. The airway response to single dose challenge with MBS was expressed as maximum fall in FEV₁% and AUC in litres per minute for change from post-saline FEV₁ >20 min. An RI was derived by expressing the difference between AUC for the first and second challenge as a percentage of the values of the first challenge. Baseline FEV₁, the maximum fall in FEV₁% and AUC for the first and second challenge and the RI were compared within subjects by student's paired t-test. Maximum fall in FEV₁, AUC and RI for drug and placebo are presented as mean \pm SEM with 95% confidence interval (CI) of the difference between the two treatments.

It was calculated that with ≥ 9 subjects receiving both treatments, the study would have 95% power to detect a change in RI between two treatments of 15% (given an inter-subject standard deviation for repeat estimation of RI of 12% [3]).

Results

Baseline data from screening visits

Challenge with the two single doses of MBS on the second screening visit caused a mean maximum fall in FEV₁ of 26 \pm 2.6% and 16 \pm 2.4% after the first and the second challenge, respectively (95% CI 6.5–13.3%, $p < 0.0001$). The AUC was 11 \pm 2.7 and 6 \pm 2 L·min⁻¹ after the first and the second challenge, respectively (95% CI 3–6.9, $p < 0.0001$). The mean RI was 55 \pm 5.6%.

Comparison of the effect of N^G-mono-methyl-L-arginine and placebo (visits 3 and 4)

There was no significant difference between baseline FEV₁ values on placebo and L-NMMA study days (3.33 \pm 0.26 L and 3.24 \pm 0.26 L respectively, 95% CI -0.054–0.22 L, $p = 0.2$).

There was no significant difference in FEV₁ after placebo and L-NMMA (3.2 \pm 0.2 L and 3.1 \pm 0.19 L respectively, 95% CI -0.02–0.14 L, $p = 0.16$).

There was no significant difference in the first challenge data between placebo and L-NMMA (mean maximum fall in FEV₁% was 26 \pm 2% and 27 \pm 2.8% respectively, 95% CI -4.9–2.3%, $p = 0.4$, AUC was 11 \pm 2.4 and 10 \pm 2.2 L·min⁻¹, respectively, 95% CI -4.7–2.1, $p = 0.6$). There was no significant difference in the second challenge data between placebo and L-NMMA (mean maximum fall in FEV₁ was

Table 1. – Effect of N^G -mono-methyl-L-arginine on refractoriness to sodium metabisulphite (MBS)

	Baseline FEV ₁ L	1st challenge		2nd challenge		RI %
		Max. fall in FEV ₁ %	AUC L·min ⁻¹	Max. fall in FEV ₁ %	AUC L·min ⁻¹	
Assessment	3.32	26	11	16	6	55
Placebo	3.33	26	11	15	5	49
L-NMMA	3.24	27	10	15	5	50
95% CI	-0.05–0.2	-4.7–2.3	-1.2–2.1	-6.16–4.6	-1.7–1.7	-20–18
p-value	0.2	0.42	0.57	0.7	1.0	0.9

Maximum fall in forced expiratory volume in one second (FEV₁), area under the percentage change in FEV₁ curve (AUC) over 20 min for the first and the second MBS challenge and refractory index (RI). Values are presented as mean, standard errors are given in text. 95% CI: 95% confidence interval of the difference between placebo and L-NMMA.

15±1.3% and 15±2.1% respectively, 95% CI -6.1–4.6%, $p=0.7$, AUC was 4.94±0.9 L·min⁻¹ for both, 95% CI for difference -1.7–1.7, $p=0.99$). There was no significant difference in the RI between placebo and L-NMMA (49±6.9% and 50±7.3% respectively, 95% CI for difference -20–18, $p=0.89$) (table 1 and fig. 1).

The effect of inhaled N^G -mono-methyl-L-arginine on exhaled nitric oxide levels

Exhaled NO levels were significantly lower after L-NMMA treatment (fig. 2). Baseline exhaled NO level was 7.6±1.0 parts per billion (ppb). Exhaled NO was 67±4.7%, 82±2.9% and 82±2.5% of baseline at 1, 15 and 60 min, respectively ($p<0.05$ at all time points).

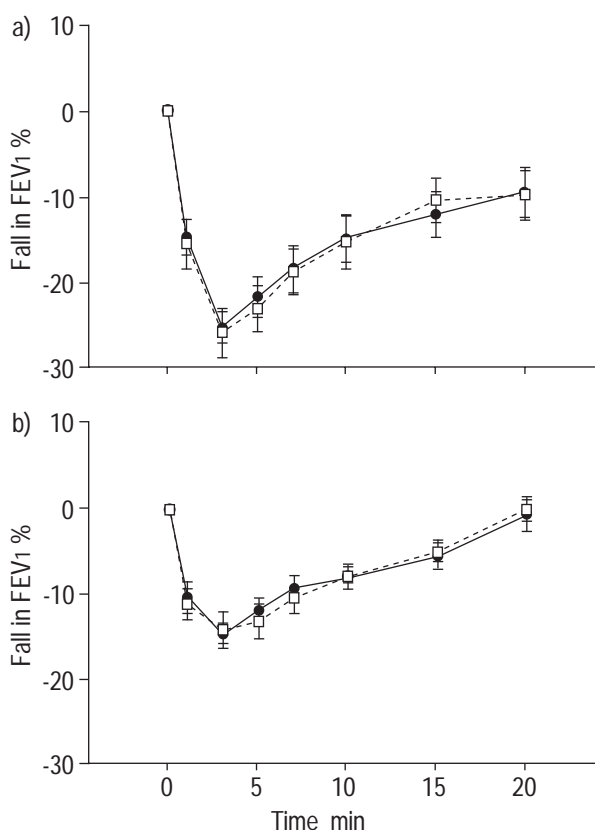


Fig. 1. – Mean±SEM change in forced expiratory volume in one second (FEV₁), expressed as percentage fall from post-saline values, in a) first and b) second sodium metabisulphite (MBS) challenges after placebo and N^G -mono-methyl-L-arginine (L-NMMA) treatment. ●: placebo; □: L-NMMA.

Discussion

The aim of this study was to determine whether the (NOS) inhibitor, L-NMMA, would inhibit MBS-induced refractoriness, thus implicating NO in the refractoriness process. It was hypothesized that release of NO from iNANC nerves during a first bronchoconstrictor challenge would protect against a second challenge. Although evidence of refractoriness to MBS-induced bronchoconstriction was shown, L-NMMA had no effect on the MBS-induced refractoriness.

The study was carefully designed to try to maximize the chance of seeing an effect. Subjects were required to show a significant degree of MBS refractoriness ($\geq 30\%$) before entry into the double-blind phase. A dose of L-NMMA which was twice as high as the dose with which RICCIARDOLO *et al.* [8] showed a marked effect on bradykinin-induced bronchoconstriction in mild asthmatic subjects was chosen. The nebulizer characteristics were comparable to those used by the latter group apart from a higher output by the current nebulizer. KHARITONOV *et al.* [9] showed that 1 mg L-NMMA significantly decreased the peak exhaled NO in healthy control subjects for ≥ 30 min. The current data confirm these findings as exhaled NO levels after inhalation of 2 mg L-NMMA led to significant inhibition of exhaled NO levels for ≥ 1 h. As all of the first MBS challenges were completed within 30 min and the second challenges proceeded within 10.5±2.3 min after the first challenge, it was likely that the dose of L-NMMA chosen was pharmacologically active in the

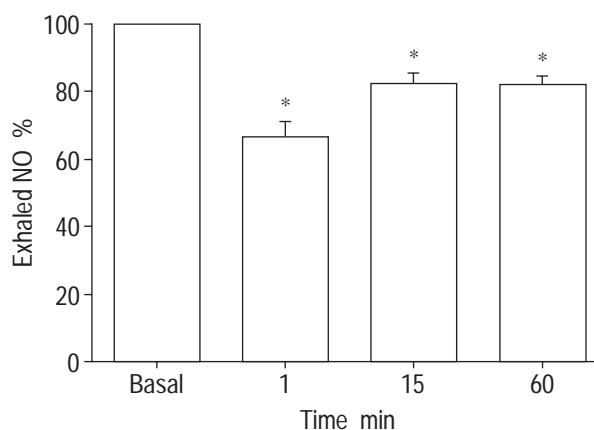


Fig. 2. – Mean±SEM change in exhaled nitric oxide (NO) levels, expressed as a percentage of baseline (basal) values, after N^G -mono-methyl-L-arginine (L-NMMA) treatment. *: $p<0.05$ compared to baseline.

airways during at least the first MBS challenge when it was postulated that NO would be released and could subsequently cause refractoriness to further challenge. This study investigated subjects who were not taking inhaled steroids, since steroids can suppress inducible (i)NOS induction [10]. This study had sufficient power to detect a change of RI of 15% in a group of subjects who had an RI of 55% at baseline. The authors therefore feel confident in concluding from this study that NO is unlikely to play a major role in MBS refractoriness.

L-NMMA did not alter resting airway calibre suggesting that NO production is not functionally important in regulating resting airway tone. This agrees with previous findings that inhaled NOS inhibitors do not alter baseline airway calibre in asthmatic subjects [11, 12]. The design of the study also enabled the investigators to comment on the effect of L-NMMA on the response to MBS *per se*. It was found to have no effect. This contrasts with studies showing that L-NMMA increases bronchoconstriction induced by bradykinin and methacholine in mild asthmatic subjects [8] although a further study in severe asthmatics showed no effect [13] and suggests that NO production may occur during bradykinin and cholinergic-induced contraction but not MBS-induced contraction.

In conclusion, it has been shown that repeated sodium metabisulphite challenge leads to refractoriness in subjects with mild asthma. The lack of an effect of N^G -methyl-L-arginine suggests that endogenous nitric oxide synthesis does not contribute to baseline airway calibre, airway responsiveness to sodium metabisulphite nor sodium metabisulphite-induced refractoriness.

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