

Effect of montelukast pretreatment on inducible nitric oxide synthase mRNA expression in the lungs of antigen-challenged allergic mice

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Summary

Background Growing evidence suggests that inducible nitric oxide synthase (iNOS) is the main source of the high output of exhaled nitric oxide (NO) in asthma. Treatment of asthmatic patients with glucocorticoids reduces high levels of exhaled NO mainly by inhibiting the transcription of iNOS. A similar reduction in exhaled NO was recently observed in patients treated with the leukotriene receptor antagonists, but the exact interaction between these drugs and iNOS remains obscure.

Objective The purpose of this study was to evaluate the effect of a leukotriene receptor antagonist, montelukast, on the expression and activity of iNOS in a murine model of allergic asthma.

Methods Twenty-four BALB/c mice were sensitized to OVA and were equally divided into 3 groups (Groups 1–3). Eight additional mice were sham sensitized and served as a negative control group (Group 4). Group 1 received montelukast 1 mg/kg/day in their drinking water, Group 2 received dexamethasone 1 mg/kg/day in their drinking water and Groups 3 and 4 received plain tap water. After 1 week, the animals were challenged by inhalation of OVA and, 3 h later, they were killed and their lung cells were isolated by enzymatic tissue digestion. NO generation was measured by a Griess assay, and iNOS mRNA was studied by RT-PCR.

Results A significant increase in iNOS mRNA expression and in NO generation was evident after allergen challenge compared with the controls. Pretreatment with montelukast mildly decreased NO production without producing a concomitant significant decrease in iNOS mRNA expression.

Conclusion: Unlike pretreatment with glucocorticoids, we failed to find compelling evidence for a major role for montelukast treatment in the modulation of iNOS mRNA in a murine model of acute asthma.

Keywords asthma, glucocorticoids, iNOS, leukotriene, mice, montelukast, mRNA, nitric oxide, RT-PCR

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Introduction

Nitric oxide (NO) is a short-lived, effector molecule that participates in many physiological processes, including vasodilatation, neurotransmission and host defence [1]. Endogenous NO is normally present in the exhaled air of animals and humans [2]. In asthma, a disease characterized by bronchial obstruction and inflammation, the NO level is increased in exhaled air and its level correlates with the course and severity of the disease [3–5]. NO is derived solely from the cationic amino acid L-arginine via the enzyme NO synthase (NOS). The three NOS isoforms that have been identified differ in tissue distribution, calcium dependency and activity [1]. In asthma, the main source of the high output of exhaled NO is the inducible NOS (iNOS). Upon stimulation by diverse cytokines, this enzyme produces large amounts of NO

for an extended period of time, in contrast to the constitutive enzymes [6–11]. Although the precise role of NO in the pathogenesis of asthma is still obscure, its role is of enormous importance. Indeed, NO is now considered as a marker of airway inflammation in asthma [12].

In both animal models of asthma and in asthmatic patients, glucocorticoids were found to reduce airway reactivity and airway eosinophilic infiltration, a reduction that was highly correlated with NO production in a dose-dependent manner [13–15]. Many studies have confirmed the fact that steroids inhibit iNOS expression and activity [9, 11, 14, 16–19], and the levels of both NO and its stable metabolites NO₂ and NO₃ (NO_x) are considered as markers of both activity and therapy in asthma [12].

In a murine model of asthma, a number of disease manifestations, including infiltration of inflammatory cells, particularly eosinophils, loss of airway structural integrity and airway obstruction, were markedly attenuated in iNOS knockout mice compared with wild-type animals [8]. iNOS expression in airway epithelium has recently been found to be

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significantly higher in untreated patients than in steroid-treated asthmatics or healthy individuals, which further suggests that iNOS may represent a target for glucocorticoid treatment in patients with asthma [11].

Cysteinyl leukotrienes (CysLTs) are other mediators that were found to be increased in asthma. They are inflammatory mediators produced by LTC₄ synthase from the biologically inactive precursor LTA₄, a product of 5-lipoxygenation of arachidonic acid [20]. In asthma, most of the biological reactions of CysLTs, including bronchospasm, plasma exudation, vasoconstriction, mucus secretion and eosinophil recruitment are mediated through interaction with a CysLT receptor. Drugs that block the production of CysLT and drugs that antagonize their receptors (such as montelukast) are currently used for the treatment of bronchial asthma [20–22].

In addition to their anti-bronchoconstrictor activity, they were claimed to have anti-inflammatory properties [20–22]. In murine models of asthma, treatment with LTRA diminished bronchial hyper-reactivity and attenuated microvascular leakage eosinophilia, remodelling and other features of inflammation, findings that support a role for CysLTs in these processes [23–27].

A relationship between LTRA and NO in asthma has recently been suggested in several reports. It was found that the administration of these drugs reduces NO production and prevents the expected increases in exhaled NO, while reducing the reduction of inhaled corticosteroid dose. The mechanism of this reduction is still obscure [28–32].

The current study was designed to clarify the role of leukotriene antagonists, specifically, montelukast, on NO production in asthma. We investigated its influence on the expression and activity of iNOS in a murine model of asthma. We assessed iNOS mRNA expression in the lungs of OVA-sensitized mice 3 h following airway allergen challenge and also examined the influence of pretreatment with LTRA montelukast on iNOS expression and NO_x generation compared with non-treated controls and dexamethasone-treated mice.

Materials and methods

Animals

All experiments described in this article were approved by our local ethics committee for the use of laboratory animals. Thirty-two female BALB/c mice, 10–12 weeks of age and free of murine-specific pathogens, were maintained on an OVA-free diet, with food and water provided *ad libitum*.

Study design

Twenty-four mice were sensitized to OVA and were equally divided into three groups (Groups 1–3). Eight mice were sham sensitized and served as a negative control group (Group 4). Group 1 received montelukast 1 mg/kg/day in their drinking water, Group 2 received dexamethasone 1 mg/kg/day in their drinking water and Groups 3 and 4 received plain tap water. After 1 week, the animals were challenged by inhalation of OVA and, 3 h later, they were killed and their

lungs were removed. iNOS mRNA expression and activity were assessed by RT-PCR and measurement of *ex vivo* NO_x production.

OVA sensitization and airway challenge

All the —24 mice (Groups 1–3) were sensitized on days 1 and 7 by intraperitoneal injection of 100 µg OVA (Grade V; Sigma Chemical Co., St Louis, MO, USA) emulsified in 8 mg aluminium hydroxide (Sigma Chemical Co.) in a total volume of 100 µL. The negative, sham-sensitized control mice (Group 4) received an equal volume of physiologic saline containing the same amount of aluminium hydroxide. On day 14, treatment was started with montelukast (Group 1) or dexamethasone (Group 2). On day 21, after 1 week of therapy, all 32 mice were challenged by OVA 5% in phosphate buffer solution (PBS). The aerosol was performed in a Plexiglas exposure chamber coupled to a jet nebulizer driven by compressed air at a flow rate of 6 L/min.

Intradermal skin testing

Mice were tested for immediate active cutaneous hypersensitivity reactions by intradermal skin test 1 week after a second sensitization with OVA. The skin of the belly was shaved under light anaesthesia with sodium brevital (10 mg/dL). For each skin test, 20 µL OVA diluted in PBS (500 µg/mL) was injected intradermally with a 30-gauge needle while the skin was stretched taut. An injection of PBS was used as a negative control and histamine as a positive control. The weal reactions were assessed 20 min following the injection. A reaction was defined as being positive if the weal diameter was greater than 3 mm in any direction.

Assessment of hypersensitivity responses

Symptoms of allergic reaction were evaluated by using a scoring system as follows: 0 = no signs, 1 = scratching and rubbing around the nose, 2 = increased respiratory rate, 3 = laboured inspiration or cyanosis around the mouth, tongue or tail, 4 = no activity or convulsions, 5 = death.

Lung mincing and digestion

After induction of general anaesthesia with sodium brevital (10 mg/dL), the chest wall was opened. The pulmonary vasculature was washed by slow injection of PBS into the heart until the lungs became white, after which they were dissected from the chest and mediastinal structures and weighed. The left lung was used for cell typing and measurement of NO generation and the right lung was used for the determination of iNOS mRNA expression.

The left lung was cut into small pieces and tissue digestion was performed by a 20-min incubation in 10 mL enriched RPMI 1640 medium (RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 10% heat-inactivated fetal calf serum) containing 20 U/mL collagenase (Sigma). The cells were separated from tissue by filtration: following incubation, the tissue was minced to a fine paste and then passed through a 75 µm stainless-steel sieve (Sigma). The resulting material was washed three times and pelleted by centrifugation at 400 g for 1 min, pooled at the end of

incubation and counted on a haemocytometer. The supernatant was collected and stored at -80°C for NO_x measurements. Slides were prepared by centrifuging 600 000 cells in an enriched RPMI 1640 for 5 min at 250 *g* in a cytocentrifuge cytospin-2 (Shandon Cheshire, England). The cellular differential was assessed on Wright–Giemsa-stained slides by counting 200 cells under oil-immersion microscopy.

Ex vivo NO production assay

The isolated lung cells from all mice were resuspended in 1 mL Dulbecco's modified Eagle's medium (DMEM) supplemented with L-arginine (2 mmol/L) and calf serum (10% vol). The medium was gassed with 95% O_2 /5% CO_2 . The cell suspension was then incubated and shaken at 37°C for 4 h, after which the incubated aliquots were centrifuged at 1600 *g* for 15 min at 4°C . The supernatant was collected and frozen at -20°C until it was assayed for the stable end products of NO (NO_2 and NO_3). The background NO_2 and NO_3 levels in the medium before incubation were subtracted from each sample result. The pellet was extracted for the determination of tissue weight.

Nitrite assay

Nitrites were measured by the addition of 50 μL freshly prepared Griess reagent (1% sulphanilic acid in 5% H_3PO_4 and 0.1% *N*-1-naphthyl ethylene diamine dihydrochloride) in double-distilled H_2O to 50 μL of a supernatant of sample from each of the experimental groups. Incubation was carried out at room temperature for 20 min, followed by spectrophotometric readings at 546 nm. The nitrite concentrations were determined from a linear standard curve between 1 and 150 $\mu\text{mol/L}$ sodium nitrite. Assays were performed in triplicate [33].

Nitrate assay

The nitrate content in the medium was measured by a bioassay with a modification of Bartholomew's method [34]. To reduce nitrate to nitrite, nitrate reductase was induced from *Escherichia coli*. The bacteria were grown for 18 h under anaerobic conditions after being washed, resuspended in PBS and stored at -70°C until use. Nitrates were measured with the Griess reagent as described in the nitrite assay.

Detection of iNOS mRNA

The total mRNA was extracted from all isolated lung cell incubates. For RT-PCR analysis, 10 μg RNA was reverse transcribed with 10 μL of $5\times$ buffer, 5 μL dithiothreitol (5 mmol/L), 10 μL deoxyribonucleoside triphosphate (2.5 mmol/L), 0.7 μg oligo-dT and 1 μg of reverse transcriptase in a total volume of 50 μL . The reaction was terminated by heating the samples at 65°C for 10 min. PCR amplification was performed in a reaction volume of 100 μL with a thermal cycler. Ten microlitres of cDNA was added to the reaction mix, which contained 10 μL of $10\times$ buffer, 2.5 μL deoxyribonucleoside triphosphate (2.5 mmol/L) and 100 pmol of each of the oligonucleotide primers in the presence of 0.5 μL *Taq* DNA polymerase (1.25 U). The primers used for iNOS were 5'-GTGTTCCACCAGGAGATGTTG-3' sense (representing

amino acids 469–475) and 5'-CTCCTGCCACT-GAGTTCGTC-3' antisense (representing amino acids 653–659) of murine iNOS [35].

Thirty-five cycles were performed under the following PCR conditions: 95°C for 1 min (denaturation), 54°C for 1 min (annealing), 72°C for 2 min (extension) and 72°C for 7 min (final extension). Fifteen microlitres of PCR products were analysed by gel electrophoresis on a 1% agarose gel stained with ethidium bromide.

To assess the adequacy of the cDNA and the efficiency of the RT-PCR system, rat GAPDH primers were used as RT-PCR controls under the same conditions. The cDNA of GAPDH was amplified with the following primers: 5'-AATGCATC CTGCACCACCAA-3' (sense) and 5'-GTAGCCATATT CATTGTCATA-3' (antisense) [35]. The steady-state mRNA levels are expressed in arbitrary units as the ratio of iNOS-to-GAPDH expression.

Statistical analyses

The values are expressed as mean $\pm$ SEM. Data from the various experiments were subjected to ANOVA, and $P < 0.05$ was considered to be statistically significant.

Results

All 24 sensitized and challenged mice (Groups 1–3) developed an allergen-specific immediate cutaneous response to the intradermal injection of OVA as well as an accumulation of basophiles in lung tissue and symptoms of allergic reaction. No responses were observed in the eight sham-sensitized control mice (Group 4) after intradermal injection of OVA or airway challenge.

iNOS mRNA expression

The PCR products amplified with iNOS primers showed clear bands at predicted sizes in the lung cells. These bands were absent in the products obtained with RNA without RT, indicating that these bands had originated from mRNA and not from genomic DNA.

The GAPDH signal at 515 base pairs was, as predicted, consistently the same in all the tissue RNAs, indicating the specific and limited effect of OVA and of the various treatments on tissue mRNA and, as such, it could be used as an internal standard for comparing the intensities of the iNOS mRNA signals.

A mild degree of iNOS expression was present in the lung cells of all control mice that were not sensitized by OVA (Group 4), but the signal was low (1.0 ± 0.16 iNOS/GAPDH). A significantly elevated signal of iNOS mRNA expression was observed in the untreated, sensitized mice (Group 3) 3 h following OVA administration (3.4 ± 0.51 iNOS/GAPDH, $P < 0.01$). Treatment with dexamethasone (Group 2) completely abolished iNOS mRNA expression (1.4 ± 0.19 iNOS/GAPDH, $P = 0.02$). In the eight montelukast-treated mice (Group 1), iNOS mRNA expression was reduced by 15%, but this was not statistically significant (2.9 ± 0.32 iNOS/GAPDH, $P = 0.09$) (Figs 1 and 2).

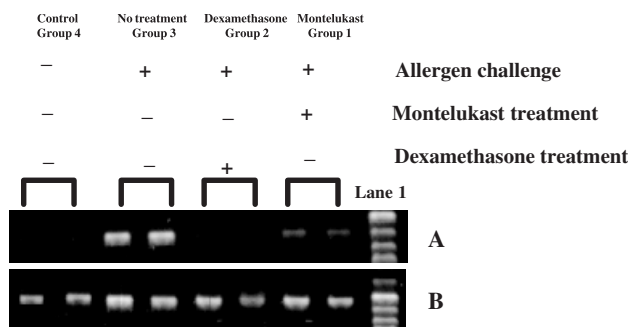


Fig. 1. RT-PCR for iNOS and GAPDH mRNA expression. RT-PCR for iNOS mRNA expression (a) and for the housekeeping gene GAPDH (b) in mice lung cells. Lane 1 contains a DNA molecular weight ladder. The four study groups are represented in duplicate. Data are from a representative experiment. Only a trace of iNOS expression was present in the lung cells of all control mice that were not challenged by OVA (Control Group 4). A significantly elevated signal of iNOS mRNA expression was observed in mice 3 h following OVA administration in the untreated mice (Group 3). Treatment with dexamethasone completely abolished iNOS mRNA expression (Group 2). Montelukast treatment mildly reduced iNOS mRNA expression (Group 1).

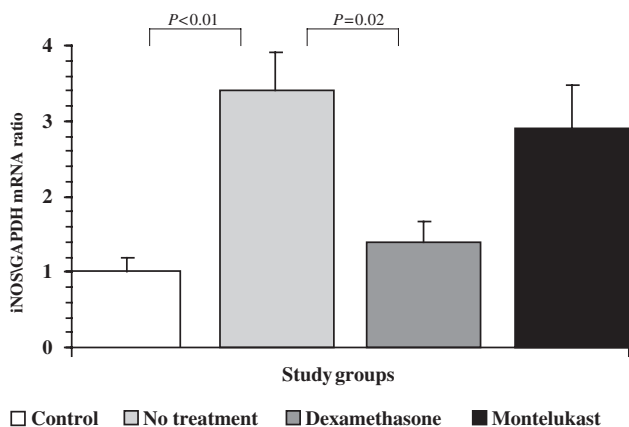


Fig. 2. iNOS-to-GAPDH mRNA expression ratio. A mild iNOS expression was present in the lung cells of all control mice that were not challenged by OVA, but the signal was low (1.0 ± 0.16 iNOS/GAPDH) (Group 4). A significantly elevated signal of iNOS mRNA expression was observed in mice 3 h following OVA administration (3.4 ± 0.51 iNOS/GAPDH, $P < 0.01$) (Group 3). Treatment with dexamethasone completely abolished iNOS mRNA expression (1.4 ± 0.19 iNOS/GAPDH, $P = 0.02$) (Group 2). iNOS mRNA expression in the montelukast-treated mice was reduced by 15%, but this did not attain statistical significance (2.9 ± 0.32 iNOS/GAPDH, $P = 0.09$) (Group 1). Each bar represents the mean ($\pm$ SEM) of eight mice. Significant differences are indicated.

NO production

To further confirm that the changes in lung cell iNOS mRNA expression were accompanied by changes in enzyme activity, we proceeded to determine iNOS activity in mice lung cells. The NO production rate in the lung cell incubates increased from 2.6 ± 0.8 pmol/h/ μ g tissue in the non-sensitized mice (Group 4) to 9.9 ± 1.7 pmol/h/ μ g tissue ($P < 0.01$) 3 h following OVA challenge in the untreated mice (Group 3). In the mice treated with either dexamethasone or montelukast, the NO_x production rate was reduced to 4.9 ± 0.9 pmol/h/ μ g tissue and 7.9 ± 1.1 pmol/h/ μ g tissue, respectively, and both changes were statistically significant ($P = 0.02$ and 0.04 , respectively) (Fig. 3).

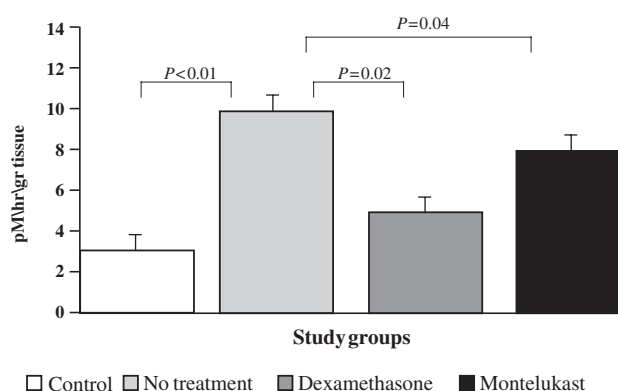


Fig. 3. *Ex vivo* NO₂ and NO₃ production. No production rate in the lung cell incubates increased from the low baseline rate of 2.6 ± 0.8 pmol/ μ g tissue before OVA (Group 4) to 9.9 ± 1.7 pmol/h/ μ g tissue ($P < 0.01$) 3 h following OVA challenge in untreated mice (Group 3). In mice treated with dexamethasone, the NO_x production rate was reduced to 4.9 ± 0.9 pmol/h/ μ g tissue ($P = 0.02$) (Group 2). In mice treated with montelukast, the NO_x production rate was reduced to 7.9 ± 1.1 pmol/h/ μ g tissue ($P = 0.04$) (Group 1). Each bar represents the mean ($\pm$ SEM) of eight mice. Significant differences are indicated.

Discussion

In the current study, we evaluated the effect of a leukotriene receptor antagonist, montelukast, on the expression and activity of iNOS in a murine model of allergic asthma. Our findings suggest that pretreatment with this LTRA in this murine model of acute allergic asthma mildly decreased NO production and did so without a concomitant significant decrease in iNOS mRNA expression.

iNOS is the main source of elevated NO production in asthma: its expression is determined primarily at the transcriptional level and is regulated by cytokine stimulation [8–12]. iNOS mRNA expression in airway epithelium is elevated in experimentally induced asthmatic animals and in asthmatic patients compared with controls, and is decreased by systemic or inhaled corticosteroids, mostly by the inhibition of gene transcription [16–19].

The investigation of the relationship between leukotrienes and NO started in the beginning of the 1990s when Pawloski and Chapnick [36] demonstrated that LTD₄ has the ability to release a labile substance, presumably NO, from the endothelium. Since then, several reports have suggested that leukotrienes may activate NO production in various tissues and states [37–39], although other studies demonstrated opposite results, depending on the cells, tissues or models that were used [40–42]. The fact that LTRA became a promising new therapy for asthma drew attention to its influence on NO production. Although some studies on asthmatic patients treated with LTRA have demonstrated reductions in exhaled NO [28–32], none demonstrated a direct influence of LTRA on iNOS expression or activity, while others have demonstrated decreased production of NO after LTRA [43–47].

NO levels and its stable products in any biological system are a sum of several synthetic and metabolic pathways. None of the studies that have thus far been published considered the possibility of the influence of LTRA on the specific contribution of iNOS on the total elevation of exhaled NO that is observed in asthma, so that the real effect of LTRA on

iNOS expression in asthma is essentially unknown. We believe that this study is the first to examine the influence of ALT on iNOS in a murine model of asthma.

Our findings that LTRA do not significantly reduce iNOS expression are supported by others. Lianos et al. [42] who investigated the regulatory interactions between iNOS and eicosanoids in glomerular immune injury, found that 5-lipoxygenase inhibition enhanced iNOS expression in a murine model of autoimmune nephritis. Holma et al. [48] reported the effects of montelukast and zileuton on iNOS in rats with experimental colitis and found no statistically significant differences between the treatment groups and the colitis control group, although high-dose montelukast reduced iNOS expression in comparison with the zileuton treatment or control group. Menard and Bissonnette [49] found that leukotriene D(4) increased alveolar macrophages NO production, but without modulating iNOS mRNA levels. Deykin et al. [50] recently found that neither leukotriene E4 inhalation nor the acute increase in human sputum eosinophils induced by leukotriene E4 inhalation resulted in increased NO production in asthma and that leukotrienes are not enough to induce NO production when given alone. It is possible that the reduced NO production that we and others had observed can be explained by an obscure indirect mechanism that does not involve iNOS induction, but rather reflects a non-specific influence of montelukast treatment on the degree of inflammation or various cytokines secretion. Using a model of acute asthma, we tried to overcome the chronic inflammatory or structural changes of persistent asthma that can influence NO production and to focus upon the immediate effects of LTRA treatment on iNOS expression.

We still do not know whether NO is a foe or a friend in asthma or whether high levels of NO in asthma are merely surrogate markers for inflammation or dose modulation of NO production, activity or metabolism should be a target for therapy in asthma.

The findings of this study indicate that, in contrast to glucocorticoids, LTRAs do not influence iNOS, the major source of NO in asthma. The meaning of these findings awaits elucidation.

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