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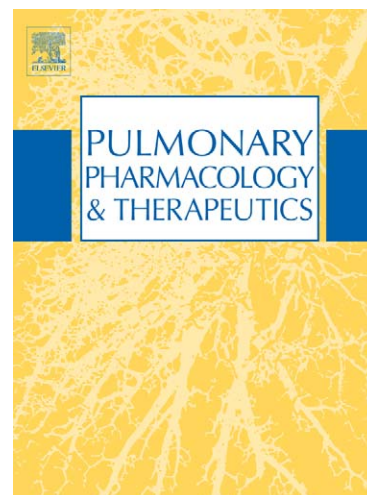
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## Montelukast exerts no acute direct effect on NO synthases

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## Abstract

The cysteinyl leukotrienes (CysLTs) LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub> are potent proinflammatory lipid mediators that play a central role in inflammation, contraction and remodelling of airways observed in asthmatics. Montelukast, a competitive inhibitor of the cysteinyl leukotriene-1 (CysLT<sub>1</sub>) receptor attenuates asthmatic airway inflammation, contraction and remodelling. As a number of studies have shown that montelukast reduced exhaled nitric oxide (NO) levels, a marker of inflammation that correlates with the severity of asthma, we investigated whether or not a direct inhibition of NO synthase (NOS) by montelukast takes place.

In an *ex-vivo* rat lung perfusion and ventilation model the NOS-dependent vasodilation effect after lipopolysaccharide (LPS) infusion was assessed with and without montelukast. Functional organ bath studies using isolated aortic rings from the same species aimed to assess effects of montelukast on the inducible and endothelial NOS isoenzymes (i- and eNOS) as well as on iNOS expression. Neuronal NOS (nNOS) was assessed by field stimulated rabbit corpus cavernosum, and isolated human iNOS enzyme activity was assessed for potential inhibition.

Montelukast failed to cause vasoconstriction in LPS challenged rat lung, or to inhibit i- and eNOS activity as well as iNOS expression in aortic rings from the same species. Also the assays for nNOS in rabbit corpus cavernosum and on isolated human iNOS enzyme gave no evidence for a direct inhibition by montelukast in physiological and supraphysiological concentrations up to 10<sup>-4</sup> M.

We therefore conclude that montelukast has no acute NOS inhibitor action. Its effect on exhaled NO is therefore probably indirectly mediated by a modulation of the asthmatic airway inflammation.

**Keywords:** Montelukast; Nitric oxide synthases; Leukotrienes; Lung vascular resistance

## 1. Introduction

The leukotrienes LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub>, collectively called cysteinyl leukotrienes (CysLTs), are potent proinflammatory lipid mediators derived via the 5-lipoxygenase pathway from arachidonic acid and play a central role in inflammation, bronchoconstriction, oedema formation and airway remodelling of asthmatics (1-4). The selective antagonist of the G protein-coupled cysteinyl leukotriene-1 (CysLT<sub>1</sub>) receptor, montelukast, has been shown to have a high binding affinity at LTD<sub>4</sub> receptors (IC<sub>50</sub> 9x10<sup>-10</sup> M; (5-7) and to functionally to antagonize LTD<sub>4</sub>-evoked contraction of guinea-pig trachea with high potency (pA<sub>2</sub> 9.7; (8)). Pharmacodynamically it mainly acts by blocking poiesis and chemoattraction of inflammatory cells like eosinophils, but also effects on cytokine and growth factor patterns and actions during the process of airway inflammation have been described (4;9-11). However, not all aspects of actions of the CysLT<sub>1</sub> receptor antagonists are fully understood, as their mode of action might be more complex than previously assumed (4;12-14).

In a study with asthmatics, LTE<sub>4</sub> inhalation did not increase exhaled nitric oxide (NO) content, despite occurrence of airway narrowing and sputum eosinophilia (15) and a concomitant expression of NO synthases (NOS) in eosinophils (16;17), suggesting a more complex relationship between NO production and the CysLTs during eosinophilic airway inflammation. A number of studies in asthmatic subjects treated with montelukast have shown that such LTD<sub>4</sub> receptor blockade is associated with lower levels of exhaled NO (18-27). In a murine allergic asthma model in which inflammation was induced by injection of ovalbumin, no evidence for a change in inducible NOS (iNOS) mRNA levels following montelukast treatment was observed (28). However, lung iNOS expression was modulated in a rat allergic inflammation model, but activity remained unchanged. In contrast, montelukast as well as dexamethasone significantly decreased iNOS expression and activity in a probably CysLT-free assay of cytokine stimulated rat lung epithelial cells, suggesting that montelukast might be able to directly or indirectly modulate iNOS function, at least in chronic models, enabling pathways to be detectable which are not related to its acute and known action as a LTD<sub>4</sub> receptor antagonist (29).

The obvious question arising from these studies is whether such a relationship between CysLTs and exhaled NO is due to a direct effect of montelukast on NO levels via NOS inhibition, or whether such effect is indirectly mediated in response to its anti-inflammatory activity, or both. Since the structures of known NOS inhibitors are heterogeneous, a theoretical prediction of a potential NOS inhibition by montelukast is virtually impossible. We

addressed that issue by using different experimental *in vitro* approaches: First, the interaction between montelukast and increased NO levels after lipopolysaccharide (LPS) challenge was studied in an *ex-vivo* CysLT-independent rat lung perfusion and ventilation model, which offers the detection of NOS-mediated changes in lung vascular resistance (30-32). Montelukast was used at a concentration of  $3 \times 10^{-8}$  M, which is more than 100-fold higher than its potency to antagonize LTD<sub>4</sub>-evoked trachea contraction (pA<sub>2</sub> 9.7; (8)). Furthermore, functional *in vitro* experiments on aortic rings from the same species were performed to assess any direct inhibitory effect of montelukast on the inducible and endothelial isoenzyme (i- and eNOS) as well as the possibility to inhibit iNOS expression (33-35). Additionally, field stimulated rabbit cavernosum strips were used to exclude inhibition of neuronal NOS (nNOS)(36;37). Finally, montelukast was investigated for its potential inhibition of isolated human iNOS enzyme (38). The highest concentrations of montelukast investigated in these assays (up to  $10^{-4}$  M) significantly exceeded those generally measured as plasma concentrations ( $3 \times 10^{-8}$  M) following oral administration of 10 mg/day montelukast sodium as therapeutic regimen (39).

## 2. Materials and methods:

### 2.1. Isolated perfused rat lung: Vascular resistance

Lungs of Wistar rats (female, 200–250 g; Harlan-Winkelmann, Borcheln, Germany) were isolated after terminal intraperitoneal anaesthesia with 160 mg/kg pentobarbital sodium and ventilated and perfused as previously described in detail (30;31;40). Wistar rats were chosen despite their relative insensitivity to LTD<sub>4</sub> as the experimental setting focused on NO synthase and not on CysLT effects and was established with this strain. Lungs were perfused at constant hydrostatic pressure (12 cm H<sub>2</sub>O) through the pulmonary artery with Krebs-Henseleit solution containing 2% BSA, 0.1% glucose, and 0.3% HEPES at 37°C, which resulted in a flow rate of approximately 35 ml/min. The pH of the perfusate before entering the lung was kept at 7.25 to 7.35 by automatic bubbling of the buffer with CO<sub>2</sub> as soon as the pH exceeded this range. The total amount of recirculating buffer was 100 ml. The relationship between vascular pressure P<sub>V</sub> and flow Q is given by the equation

$$P_V = R_V \times Q$$

where  $R_V$  represents vascular resistance. In case of present constant-pressure perfusion ( $P_V = \text{constant}$ ), perfusion flow rate  $Q$  is measured to calculate vascular resistance  $R_V$ . The lungs were suspended by the trachea and ventilated by negative pressure ventilation (inspiratory pressure:  $-7 \text{ cm H}_2\text{O}$ , expiratory pressure:  $-2 \text{ cm H}_2\text{O}$ ) with 80 breaths/min resulting in a tidal volume of approximately 2 ml. Every 5 min a deep inspiratory breath ( $-20 \text{ cm H}_2\text{O}$ ) was performed. A weight transducer was integrated into the chamber lid and allowed continuous measurement of lung weight. Additionally, parameters of lung mechanics were analyzed by applying the following formula

$$P = 1/C_L \times V_T + R_L dV/dt$$

where  $P$  is chamber pressure,  $C_L$  pulmonary compliance,  $V_T$  tidal volume, and  $R_L$  airway resistance, reflecting the pressure that forces the volume  $V$  at the velocity  $dV/dt$  into the lung. All parameters were normalized to the time point at the end of the preconditioning perfusion, which was at 40 min post-lung isolation. Perfusion and ventilation was continued for another 60 min. Data were recorded on a computer using the Matlab Software package (MathWorks, Inc., Natick MA, USA).

## 2.2. Isolated rat thoracic aorta: iNOS and eNOS inhibition

Thoracic aortae were obtained from Wistar rats (male, 250-300 g; Charles River, Kisslegg, Germany) killed by cervical dislocation. Aortic rings of 1.5 mm length were mounted under 1 g tension in 10 ml organ baths filled with warmed ( $37 \text{ }^\circ\text{C}$ ), oxygenated (95%  $\text{O}_2/5\% \text{CO}_2$ ) Krebs solution (mM): NaCl 120.0, KCl 5.5,  $\text{CaCl}_2$  2.5,  $\text{MgCl}_2$  1.2,  $\text{NaH}_2\text{PO}_4$  1.2,  $\text{NaHCO}_3$  25.0 and glucose 11.0, additionally containing  $10^{-5} \text{ M}$  indomethacin for the experiments of iNOS inhibition. Isometric tension changes were measured by force-displacement transducers. In case of iNOS inhibition, the endothelium was removed by gently rubbing the intima surface before cutting the vessel into rings, which were then placed in Petri dishes containing RPMI-1640 medium with LPS (200 ng/ml, *Salmonella abortus equi*) and incubated at  $37 \text{ }^\circ\text{C}$  for 16 h. Aortic rings were considered denuded when a maximal concentration of the muscarinic receptor agonist, arecaidine propargyl ester (APE,  $3 \times 10^{-6} \text{ M}$ ), caused relaxation of less than 5% of the precontracted vessel.

Rat aortic rings were precontracted by  $3 \times 10^{-7} \text{ M}$  phenylephrine ( $\text{EC}_{80-90}$  of its own maximal effect), after that  $3 \times 10^{-6} \text{ M}$  APE ( $\text{EC}_{100}$ ) was added to cause rapid relaxation of the tissue within 5 min, before the cumulative administration of the test drug was started to evoke

contraction due to inhibition of eNOS. Likewise, for measuring iNOS inhibition, LPS-treated aortic rings were challenged with  $3 \times 10^{-7}$  M phenylephrine, which caused a partial contraction (5-10% compared with untreated tissue). Thereafter, due to inhibition of iNOS, cumulative administration of test drugs restored the contraction of the hyporeactive tissue. The non-selective NOS inhibitor 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT) at  $10^{-5}$  M was finally added to define the maximal contractile effect of test drug related to that elicited by AMT (100%). All results were expressed as a percentage of the AMT-induced maximal response (34;35;38).

### 2.3. Isolated rat aorta: iNOS expression

Similarly, the inhibition of iNOS expression can be assessed in rat aortic rings rendered hypoactive with LPS alone (200 ng/ml, *Salmonella abortus equi*) and by co-incubation with the test drug during LPS exposure (16 h). After this treatment, the restoration of contractility of the tissue to cumulatively administered phenylephrine ( $10^{-8}$ – $3 \times 10^{-5}$  M) by active test drugs, e.g. dexamethasone ( $10^{-8}$  and  $10^{-7}$  M) or cycloheximide ( $10^{-6}$  and  $10^{-5}$  M), can be assessed in comparison with concomitantly run control rings untreated with LPS and test drug (41).

### 2.4. Field stimulated rabbit corpus cavernosum: nNOS inhibition

Adult New Zealand White rabbits (male, 2.5–3.0 kg; Dunkin Hartley, Charles River, Kisslegg, Germany) were sacrificed by intravenous pentobarbital injection (60 mg/kg) and exsanguination. Four longitudinal strips (1.5x1.5x6 mm) were dissected from each corpus cavernosum and mounted in organ baths under a resting tension of 1 g to measure isometric tension changes in Krebs solution (mM: NaCl 118.3, KCl 4.7, CaCl<sub>2</sub> 1.9, MgSO<sub>4</sub> 1.2, KH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25.0, Na-EDTA 0.03 and glucose 11.1), additionally containing  $10^{-6}$  M atropine and  $5 \times 10^{-6}$  M guanethidine to exclude a possible participation of cholinergic or adrenergic responses, respectively, to electrical field stimulation (EFS). The nutrient solution was aerated with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub> maintaining pH 7.4 at 37 °C. The strips were precontracted by  $10^{-5}$  M phenylephrine (EC<sub>80-90</sub> of its own maximal response); after that, relaxant nonadrenergic, noncholinergic (NANC) responses were evoked by EFS (15 V, 0.3 ms, 4 Hz, for 5 s, every 2 min) using a pair of platinum electrodes, one located at the bottom of the organ bath and directly connected with the tissue, and the other ring electrode placed at the top of the bathing fluid. After stabilization of EFS-evoked relaxant responses within 30 to 45 min, the test drug was added in a cumulative manner. The potential inhibition of nNOS



could be followed by inhibition of tissue relaxation mediated by synthesis and release of neuronal NO (36-38).

### 2.5. Human iNOS activity assay

The enzyme reaction using isolated human iNOS was performed in 96-well microtiter F-plates at L-arginine concentrations of 3 to  $5 \times 10^{-6}$  M essentially as described previously (38;42). After adding [ $^3$ H]L-arginine (150,000 dpm/well) the assay mixture was incubated for 60 min at 37 °C after that it was stopped and analysed for [ $^3$ H]L-citrulline content in a Micro-Beta scintillation counter (PerkinElmer Wallac, Turku, Finland).  $-\log(\text{IC}_{50})$ M values for test drugs were calculated from concentration-response curves by Prisma 3.0 (GraphPad Inc., San Diego, CA).

### 2.6. Drugs and chemicals

Montelukast was a gift from Merck Frosst Canada. The NOS inhibitors AMT (2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride), L-NMMA (N<sup>o</sup>-monomethyl-L-arginine monoacetate), L-NIL (L-N<sup>o</sup>-(1-iminoethyl)lysine hydrochloride) and L-NAME (N<sup>o</sup>-nitro-L-arginine methyl ester) were purchased from Alexis (Läufelingen, Switzerland). APE (arecaidine propargyl ester) and phenylephrine hydrochloride were from Tocris (Köln, Germany). Cycloheximide and dexamethasone were from Sigma (Munich, Germany). Pentobarbital sodium was from Merial (Hallbermoos, Germany). LPS from *Salmonella minnesota* or *Salmonella abortus equi* was purchased from Sigma (Munich, Germany). Bovine serum albumin (BSA), fraction V was from Serva (Heidelberg, Germany), HEPES from ICN Biomedicals Inc. (Ohio, USA).

### 2.7. Statistical analysis

Data are given as mean and standard error of the mean (mean  $\pm$  SEM) unless otherwise indicated. In the rat lung perfusion experiments, two-way ANOVA was used to differentiate between drug effects with Bonferroni-post hoc analysis, considering  $p \leq 0.05$  as significant.

$-\log(\text{IC}_{50})$ M values for half-maximal restoration of contraction of LPS- or APE-pretreated rat aortic rings (inhibition of i- and eNOS, respectively) or inhibition of relaxant responses to EFS in rabbit corpus cavernosum (inhibition of nNOS) were calculated by a linear curve fitting model and were related to their individual maximal inhibitory effects.

### 3. Results

#### 3.1. *Isolated perfused rat lung*

The isolated perfused rat lung model was used to assess the involvement of vascular NO in response to LPS. Infusion of LPS alone (100  $\mu\text{g/ml}$  of *Salmonella minnesota*) did not increase vascular resistance normalized to the end of the preconditioning perfusion of 40 min and expressed as  $R_V/R_{V40\text{min}}$  (Fig. 1). However, when LPS was co-administered with the NOS inhibitor L-NMMA ( $10^{-4}$  M), a significant increase in vascular resistance occurred, which was maximal at 40 min after combined drug administration, demonstrating the involvement of NO synthesis to keep the vascular system dilated after LPS treatment. In contrast, a combination of LPS and montelukast ( $3 \times 10^{-8}$  M) did not cause vasoconstriction in this model, suggesting that montelukast has no inhibitory effect on NO synthesis. Furthermore, no change of vasotonus by administration of montelukast itself is observed (Fig. 1).

On the other hand, perfusion of the rat lung with LPS increased airway resistance  $R_L$ , decreased pulmonary compliance  $C_L$  and tidal volume  $V_T$ , starting at about 30 min after LPS administration (not shown). The time lag of 30 min is probably due to induction of cyclooxygenase-2 (COX-2), as supported by the ability of the COX-2 inhibitor CGP-28238 to prevent LPS-induced thromboxane release and hence bronchoconstriction in this model (31). Administration of LPS together with L-NMMA ( $10^{-4}$  M), did not affect the LPS-induced bronchoconstriction, revealing that synthesis of NO does not participate in this response. Also montelukast ( $3 \times 10^{-8}$  M) did not impair the increase in airway resistance evoked by LPS, whereas montelukast, L-NMMA or vehicle, each being administered alone, did not show any effect on airway resistance (not shown). For completion, previous findings have also shown that neither inhibition of 5-lipoxygenase with AA-861 nor blockade of  $\text{LTC}_4/\text{LTD}_4$  receptors with MK-571 prevent the LPS-induced bronchoconstriction (31).

Similarly, neither LPS alone, nor its combination with montelukast ( $3 \times 10^{-8}$  M) or L-NMMA ( $10^{-4}$  M) significantly affected the lung weight, as compared to those treated with L-NMMA, montelukast or vehicle alone (not shown).

#### 3.2. *Effect of montelukast on i- and eNOS in rat aorta and nNOS in rabbit corpus cavernosum*

The reference NOS inhibitors, L-NIL, L-NMMA and L-NAME, caused concentration-dependent reversal of contraction to phenylephrine of rat aortic rings rendered hypoactive with LPS (iNOS) or relaxed by the muscarinic receptor agonist APE (eNOS), and attenuated nitrenergic relaxations in response to EFS in rabbit corpus cavernosum (nNOS). Concentration-response curves of these compounds are depicted in Fig. 2, their  $-\log(\text{IC}_{50})\text{M}$  values being summarized in Table 1. The rank order of potency of the standard inhibitors observed for inhibition of iNOS in rat aorta (L-NIL > L-NMMA > L-NAME) was different from that observed for the compounds displayed both on eNOS in rat aorta and nNOS in rabbit corpus cavernosum (L-NAME > L-NIL > L-NMMA). As derived from their  $-\log(\text{IC}_{50})\text{M}$  values on these three NOS isoenzymes, L-NIL is selective for i- over e- and nNOS, L-NAME is selective for both e- and n- over iNOS, whereas L-NMMA behaved non-selective. However, none of the three NOS isoenzymes was inhibited by montelukast up to already supraphysiological concentrations of  $3 \times 10^{-5}$ - $10^{-4}$  M (Fig. 2).

### 3.3. *Effect of montelukast on iNOS expression*

In a further series of experiments, the contraction of the rat aorta to cumulative administration of phenylephrine ( $10^{-8}$ - $3 \times 10^{-5}$  M) 16 h after LPS treatment alone or in the additional presence of the protein synthesis inhibitor cycloheximide, the glucocorticoid dexamethasone or the test drug montelukast was investigated (Fig. 3). Phenylephrine caused concentration-dependent contraction of the tissue, which could be nearly totally prevented treatment of the tissue with LPS for 16 h. Cycloheximide ( $10^{-6}$  and  $10^{-5}$  M) in the presence of LPS, concentration-dependently restored the contractile response to phenylephrine nearly to levels obtained in untreated control tissue, whereas dexamethasone ( $10^{-8}$  and  $10^{-7}$  M) in the presence of LPS restored at least 50% of maximal contraction to the agonist. However, montelukast up to  $10^{-5}$  M (lower concentration of  $10^{-6}$  and  $10^{-7}$  M were also ineffective but not depicted in Fig. 3) when co-incubated with LPS, did not prevent the endotoxin-induced vascular hypocontractility in rat aortic rings, demonstrating its failure to inhibit the induction/expression or synthesis of NOS.

### 3.4. *Effect of montelukast on human iNOS activity*

Finally we analysed if montelukast has a direct inhibitory effect on isolated human iNOS by measuring [ $^3\text{H}$ ]L-arginine conversion to [ $^3\text{H}$ ]L-citrulline. Whereas the reference NOS inhibitors, L-NIL, L-NMMA and L-NAME, caused a concentration-dependent inhibition of

human iNOS ( $-\log(\text{IC}_{50})$ )M 6.29, 5.91 and 4.40, respectively), montelukast up to  $3 \times 10^{-5}$  M was without any inhibitory effect. Even at the highest concentration investigated ( $10^{-4}$  M), inhibition amounted to less than 20% (Fig. 4).

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#### 4. Discussion

Since the CysLT<sub>1</sub> receptor antagonist, montelukast, has been shown to reduce NO levels in exhaled air of asthmatics, the present *in vitro* experiments aimed to assess whether this drug is capable (a) to influence NO synthesis after endotoxin challenge in perfused rat lung, (b) to directly inhibit different NOS isoenzymes (i-, e- and nNOS) derived from rat, rabbit and human, and (c) to affect iNOS expression in rat vasculature.

In an *ex-vivo* rat lung perfusion and ventilation model we could demonstrate that vasodilatation in response to added endotoxin (LPS) maintained by stimulation of NO synthesis, did not change even with a more than physiological concentration of montelukast. This is consistent with effects observed with endotoxin on airways, showing that neither inhibition of 5-lipoxygenase with AA-861, nor LTC<sub>4</sub>/LTD<sub>4</sub> receptor blockade by MK-571 prevented the LPS-induced increase in airway resistance (31). Similarly, previous studies have shown that, while L-NMMA (10<sup>-4</sup> M) without LPS did not cause any change in vascular resistance in perfused rat lung as compared to controls, infusion of L-NMMA together with LPS leads to a similar production of the stable eicosanoid degradation products, thromboxane B<sub>2</sub> und 6-keto-PGF<sub>1 $\alpha$</sub> , as LPS alone (31). Therefore, changes in vascular resistance in this setting seem not to be critically dependent on the thromboxane-prostacyclin system. As a result of the present study, the relaxing effect of NO on the vascular system in rat perfused lung can be observed roughly 40-50 min after LPS administration attributable to permanent NO synthesis by inducible NOS (iNOS), which can be converted into vasoconstriction by concomitant administration of a NOS inhibitor, like L-NMMA, but not by montelukast.

In order to further corroborate these results with montelukast, additional *in-vitro* studies in thoracic aorta from the same species were performed to assess its potential inhibition of iNOS and eNOS and to affect iNOS expression in this tissue. It has been previously shown that incubation with LPS activates iNOS in rat aorta to generate NO, which induces a permanent hyporeactivity to  $\alpha_1$ -adrenoceptor agonists, and that inhibitors of iNOS are capable to restore the contractile response to the agonists to control (35). Thus, iNOS and eNOS inhibition by drugs was tested as reversal of contraction to the  $\alpha_1$ -adrenoceptor agonists phenylephrine of rat aortic rings rendered hypoactive by either overnight treatment with LPS or acutely by muscarinic receptor stimulation with APE (34). On the other hand, NO synthesized by a cytosolic, constitutive isoform of NOS (nNOS) like that found in brain

neuronal tissue, is the predominant nonadrenergic noncholinergic (NANC) relaxant neurotransmitter released in rabbit corpus cavernosum in response to EFS (36). Previously, these functional *in-vitro* assays for i-, e- and nNOS and their inhibition by unselective or isoenzyme-selective inhibitors had been compared with human isoenzymes revealing that both rat aorta and rabbit corpus cavernosum can be used to assess selectivity for inhibitors observed at human isoenzymes (34;37;38). Particularly, in rat aorta L-NIL has been characterized as being 14-fold selective for iNOS over eNOS ( $-\log(\text{IC}_{50})$  6.0 vs. 4.9), exactly matching the 17-fold selectivity assessed for human iNOS over eNOS ( $-\log(\text{IC}_{50})$  6.2 vs. 5.0), whereas L-NAME showed selectivity for eNOS over iNOS in rat aorta ( $-\log(\text{IC}_{50})$  5.6 vs. 4.0; 40-fold selectivity) and on human isozymes ( $-\log(\text{IC}_{50})$  5.7 vs. 4.4; 20-fold selectivity) [this study; (34;38)]. Furthermore, a selectivity of L-NAME for nNOS over iNOS could be demonstrated using rabbit corpus cavernosum and rat aorta (11-fold) and human isozymes (25-fold) (37).

A possible inhibition of iNOS expression or its synthesis was investigated in rat aortic rings and demonstrated by the ability of dexamethasone or cycloheximide, respectively, to partly restore contraction to phenylephrine of tissue previously treated and rendered hypoactive with LPS (41). Again, montelukast up to  $10^{-5}$  M did not show any effect. To answer the question whether montelukast has an inhibitory effect on human iNOS, we performed experiments on isolated human iNOS enzyme. Montelukast up to the highest concentration investigated of  $10^{-4}$  M does not inhibit the enzymatic activity. Likewise, experiments on respective human e- and nNOS isoenzymes yielded the same results (not shown). Taken together these assays clearly gave no evidence of any direct inhibitory effect of montelukast on NOS isoenzymes within the concentration range from  $10^{-7}$  to  $10^{-4}$  M, far exceeding that achieved during drug therapy (39). Therefore, alternative explanations for the decreased NO levels measured in expired air from asthmatic subjects after treatment with montelukast should be considered (18-27).

Montelukast has been shown to reduce iNOS expression in the lung tissue of ovalbumin challenged rat after 4 weeks of treatment while the iNOS activity remained unchanged. In addition, montelukast as well as dexamethasone significantly decreased iNOS expression and activity in a probably CysLT-free assay of rat lung epithelial cells stimulated with cytokines. This finding suggests that montelukast might be able to directly or indirectly modulate iNOS function as an anti-inflammatory compound after long time exposure to allergen challenged animals (29). This is consistent with the finding, that reduced levels of

exhaled NO can be measured only after some time of chronic treatment with montelukast, which parallels its effect of other surrogate markers of inflammation such as blood eosinophils. Thus, the effect of montelukast on exhaled NO levels observed during asthma therapy excludes direct effects on NOS enzymes, but most probably is secondary to its expected effect as antagonist of CysLTs.

Since no acute inhibitory effect on NO synthesis, neither directly on the three NOS isoenzymes, nor a suppression of the expression of iNOS was found, alternative explanations for the decreased NO levels measured in expired air from asthmatic subjects after treatment with montelukast must be considered (18-27). They may be explained by changes in quantities or sites of NO generation, binding or more distal processes. Those effects might occur later than assessed in our experimental settings, e.g. with prolonged drug administration, and might therefore be due to the drug to antagonize the effect of CysLTs which might reduce airway inflammation.

A further possibility is that CysLTs may have biological effects exceeding allergic disease. Bisgaard *et al.* could demonstrate that infants with respiratory syncytial virus (RSV) bronchiolitis profited in terms of subsequent reactive airway disease (4;43). There are a number of observations that CysLTs might have broader effects in several disease processes (44-50).

In conclusion, none of the functional *in-vitro* assays in our study gave evidence for a direct acute inhibition of the different NOS isoenzymes (i-, e- and nNOS) and of iNOS expression by montelukast, even up to high concentrations and far exceeding those observed during drug therapy (39). Its observed effect to reduce exhaled NO in asthmatics is therefore probably secondarily mediated, presumably indirectly due to effects on inflammatory mediators, cytokines, growth factors, receptors, and their partially convergent actions ultimately occurring to reduce inflammation during asthma (4).

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## Legends of the figures

Fig. 1. Change of vascular resistance normalized to that measured at 40 min ( $R_V/R_{V40 \text{ min}}$ ) in the isolated perfused rat lung after drug administration at 40 min into the perfusate. LPS (100  $\mu\text{g/ml}$ ; control  $n = 14$ ) together with L-NMMA ( $10^{-4}$  M;  $n = 3$ ) causes vasoconstriction, whereas no vasoconstriction occurs with LPS administered together with montelukast ( $3 \times 10^{-8}$  M;  $n = 4$ ). Given are means  $\pm$  SEM of  $n = 3$ -14. \*;  $p < 0.0001$  for LPS + L-NMMA vs. LPS + montelukast at the time points indicated.

Fig. 2. Cumulative concentration-response curves for NOS inhibitors in comparison with montelukast to restore contraction to  $3 \times 10^{-7}$  M phenylephrine of rat aortic rings rendered hypoactive with LPS (inhibition of iNOS, top), or relaxed with  $3 \times 10^{-6}$  M APE (inhibition of eNOS, middle), and to inhibit nitrenergic relaxation to EFS in rabbit corpus cavernosum (inhibition of nNOS, bottom). Given are means  $\pm$  SEM of  $n = 10$ -16.

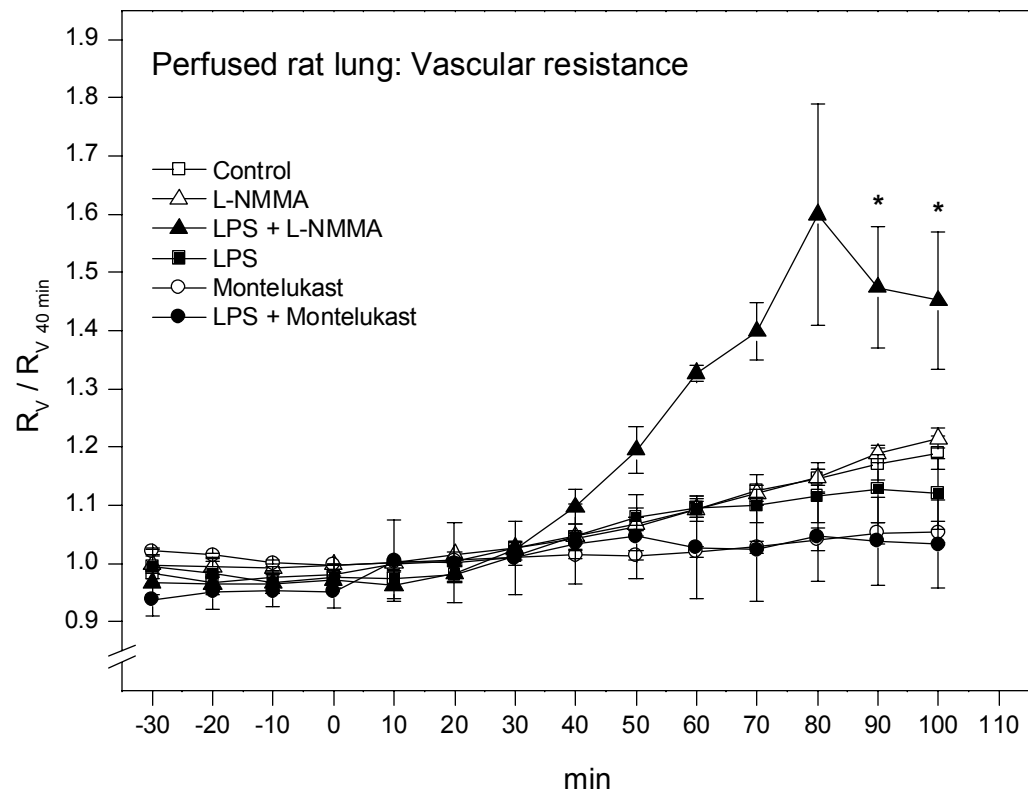
Fig. 3. Cumulative concentration-response curves of phenylephrine on untreated rat aortic rings (Control), treated for 16 h with LPS alone (LPS) or in the additional presence of either cycloheximide ( $10^{-6}$ ,  $10^{-5}$  M), dexamethasone ( $10^{-8}$ ,  $10^{-7}$  M) or montelukast (shown is the missing effect of  $10^{-5}$  M; lower concentrations of  $10^{-7}$  to  $10^{-6}$  M were also ineffective and not depicted). Given are means  $\pm$  SEM of  $n = 6$ -8.

Fig. 4. Inhibition of human iNOS by NOS inhibitors in comparison with montelukast. –  $\log(\text{IC}_{50})$ M values of 6.29, 5.91 and 4.40 were obtained for L-NIL, L-NMMA and L-NAME, respectively, whereas the value for montelukast amounted to be less than 3.00. Given are means  $\pm$  SEM of  $n = 6$  determinations.

Table 1

Potencies of drugs for inhibition of iNOS and eNOS in rat thoracic aorta and of nNOS in field stimulated rabbit corpus cavernosum. Given are  $-\log(\text{IC}_{50})\text{M}$  values (with % maximal inhibitory effect in brackets) for the drugs derived from their individual half-maximal effects (means  $\pm$  SEM, n = 10-16)

	$-\log(\text{IC}_{50})\text{M}$		
	iNOS	eNOS	nNOS
L-NIL	$6.03 \pm 0.03$ (90)	$4.90 \pm 0.03$ (94)	$4.58 \pm 0.08$ (74)
L-NMMA	$4.78 \pm 0.06$ (49)	$4.73 \pm 0.04$ (72)	$4.19 \pm 0.06$ (66)
L-NAME	$4.01 \pm 0.03$ (38)	$5.56 \pm 0.02$ (100)	$5.05 \pm 0.03$ (96)
Montelukast	<4.0	<4.5	<4.0



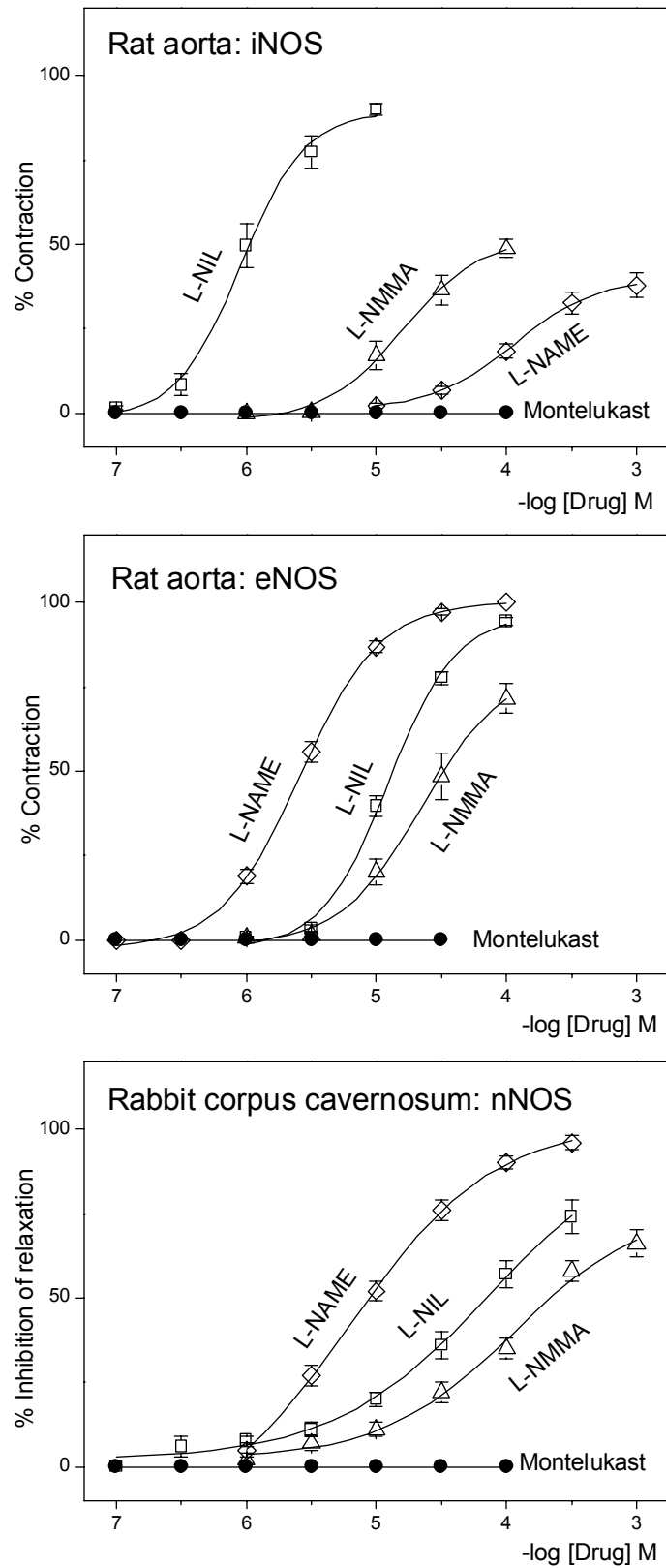


Figure 3

