Airway arginase expression and $N^\omega$-hydroxy-nor-arginine effect on methacholine-induced bronchoconstriction differentiate Lewis and Fischer rat strains

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Innate airway hyperresponsiveness (AHR) is well modeled by two strains of rat, the hyperresponsive Fischer 344 rat and the normoresponsive Lewis rat. Arginase has been implicated in AHR associated with allergic asthma models. We addressed the role of arginase in innate AHR using the Fischer-Lewis model. In vivo arginine inhibition with $N^\omega$-hydroxy-nor-arginine (nor-NOHA) was evaluated on methacholine-induced bronchoconstriction in the Fischer and the Lewis rats. Arginase activity and mRNA expression were quantified in structural and resident cells of the proximal airway tree. The effect of nor-NOHA was evaluated on cultured tracheal smooth muscle proliferation. Fischer rats exhibited significantly greater changes in respiratory resistance and elastance in response to methacholine compared with Lewis rats. nor-NOHA reduced the methacholine-induced bronchoconstriction in the central airways of Lewis rats, while it did not change the innate AHR of Fischer rats. Lewis rats exhibited greater arginase activity in tracheal smooth muscle but a lower proliferation rate compared with Fischer rats. Smooth muscle proliferation was not affected by nor-NOHA in either strain of rats. The strain-specific arginase expression in the smooth muscle may contribute to the differences in sensitivity of the methacholine challenged airways of Lewis and Fischer rats to inhibition of arginase.

Methods

Animals. Specific pathogen-free male Fischer 334 and Lewis rats, aged 8–12 wk, were purchased from Harlan (Mississauga, ON, Canada) and housed in a conventional animal facility at McGill University. All of the protocols were approved by an institutional animal care committee, and procedures were in conformity with the guidelines of the Canadian Council on Animal Care.

Measurements of airway responsiveness. Rats were sedated with an intraperitoneal (ip) injection of xylazine hydrochloride (8 mg/kg) and anesthetized with pentobarbital (30 mg/kg ip). Subsequently, the animals were tracheostomized and connected to a small-animal ventilator (Flexivent, Scireq, Montreal, QC, Canada). Muscle paralysis was induced with pancuronium bromide (0.2 mg/kg iv). The rats were ventilated in a quasi-sinusoidal fashion with 60 breaths/min, a tidal volume of 4 ml, a respiratory rate of 50 breaths/min, and a 60% inspiratory fraction.

Airway hyperresponsiveness (AHR) is a defining feature of asthma and is a reflection of an enhanced capacity of the airways to narrow in response to exposure to bronchoconstrictors. The basis for AHR is not yet known, but in some instances may be acquired through repeated exposure of sensitized subjects to allergens (7) or following exposures to strong irritants (5). Some asymptomatic subjects have AHR, indicating that it may precede the development of symptomatic asthma (3). Such innate AHR has been studied through large- and small-animal models (9, 11, 19, 22, 29) with a view to providing insights into the risk that it represents for the development of disease.

The presence of innate AHR has been documented in several species, including Fischer 344 rats (28). These rats have higher responsiveness to methacholine (MCh) and to serotonin compared with the control strain Lewis. Airway smooth muscle (ASM) is more abundant in the airways of these animals and has been postulated to be the basis for AHR (10). There appear to be alterations in the function of ASM that contract more completely and more rapidly in airways studied within cultured lung slices (28), and the cells have an increased contractile response in vitro (1). Also, cultured ASM cells from Fischer rats proliferate more rapidly on treatment with mitogens, such as fetal bovine serum (FBS) and platelet-derived growth factor (30), analogous to human asthmatic ASM cells (14).

The molecular basis for the innate AHR in the rat appears to be complex and to potentially involve multiple pathways that are likely coordinately regulated to result in a more contractile phenotype in the Fischer 344 rat smooth muscle. Cultured ASM cells demonstrate exaggerated calcium signals in response to agonists such as serotonin. Lewis rats are also more sensitive to the bronchorelaxant effects of sodium nitroprusside, a nitric oxide (NO) donor as well as to $N^\omega$-nitro-$\omega$-arginine methyl ester (l-NAME), a NO synthase (NOS) pseudosubstrate, whereas Fischer rats are resistant to its effects (12, 13). Decreased l-arginine bioavailability has been shown to be associated with increased arginase activity in asthma (21), and blockade of arginase has been demonstrated to inhibit allergendriven experimental asthma in animal models (18, 25, 27). Since l-arginine is a common substrate for NOS and arginase, arginase might contribute to smooth muscle responsiveness in at least two different ways. It could first reduce l-arginine bioavailability for NOS and limit the production of NO-induced ASM relaxation. Secondly, arginase generates polyamines through the actions of ornithine decarboxylase, one of which, spermine, when administered to the mouse, has been shown to enhance airway responsiveness (24). We reasoned, therefore, that differences in the arginase expression profile might contribute to the observed differences in induced airway constriction in Fischer and Lewis rats.

METHODS

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volume of 8 ml, and a positive end-expiratory pressure of 2–3 cmH₂O. Cumulative doses of MCh were administered using a nebulizer (Aeroneb) and progressively doubling concentrations ranging from 4 to 128 mg/ml. In some experiments, the role of arginase on AHR was assessed on cytospin preparations (Shandon Cytospin 4, Thermo Scientific). Tracheal epithelial cells. Tracheal epithelial cells (TEC) were obtained by gentle brushing of the epithelial layer of the trachea and then immersed in the corresponding lysis buffer for RNA extraction or for arginase activity assay.

Bronchoalveolar lavage. Bronchoalveolar lavage was performed with a single instillation of 5 ml of saline solution. The total cell count was measured using a hemacytometer, and the differential cell count was assessed on cytospin preparations (Shandon Cytospin 4, Thermo Scientific, Ottawa, ON, Canada) from a count of 300 cells stained with Diff-Quik (Fischer Scientific).

RNA extraction and quantitative PCR. RNA from cultured TSMC and epithelial cells were extracted with the RNeasy-mini kit (Qiagen, Valencia, CA). Reverse transcription was performed from 250 ng of RNA with the AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies, Santa Clara, CA). Sequences of primers and amplicon size are listed in Table 1. Quantitative PCR was performed using an Applied Biosystem PCR apparatus (manufacturer and address). Each PCR reaction contained 1 μl of cDNA, 0.3 μl of both forward and reverse primers (10 μM), 3.4 μl of DNase RNase free water, and 5 μl of QuantiTect SYBR Green (Qiagen, Valencia, CA). Expression of target genes was normalized with the housekeeping gene S9 and related to a reference sample.

Table 1. Primers used for PCR

<table>
<thead>
<tr>
<th>Target Genes</th>
<th>Sequences</th>
<th>Amplicon Size, bp</th>
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<tbody>
<tr>
<td>ARG1</td>
<td>(R): CTGGGGAGCCTTTCTCTACC (F): AGGATTTCTTGGAGAGAAGGCTGC</td>
<td>101</td>
</tr>
<tr>
<td>ARG2</td>
<td>(R): GAGCATCaCCTTTGTTTCCAC (F): CATGAGGACCTGCTGACC</td>
<td>138</td>
</tr>
<tr>
<td>NOS3</td>
<td>(R): CTTGGCTCCTTGTTTCCAC (F): CATGAGGACCTGCTGACC</td>
<td>110</td>
</tr>
<tr>
<td>S9</td>
<td>(R): AGATTTCTTGGAGAGAAGGCCTGC (F): CTTGGTGGAGCTCAGCGAACAAT</td>
<td>158</td>
</tr>
</tbody>
</table>

ARG1, arginase-1; ARG2, arginase-2; NOS3, nitric oxide synthase 3; S9, ribosomal protein S9; R, reverse; F, forward.

Fig. 1. Respiratory mechanics parameters in response to provocation with cumulative doses of methacholine in Fischer and Lewis rats. Fischer rat strain exhibited a significantly higher respiratory system resistance (Rrs; A) and elastance (Ers; B) than Lewis rat. This phenomenon involves an increase of resistance in the conducting airways [Newtonian resistance (Rn); C] and an increase of tissue resistance (G; D) and tissue elastance (H; E). Values are means ± SE; n = 5–6/group. *P < 0.05.

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Arginase activity. In a first set of experiments, following bronchoalveolar lavage, the structural tissue of the airway tree was isolated by a gentle scraping of the lung parenchyma and was then homogenized in 25 mM Tris·HCl, pH 7.5, 0.5% Triton X-100 containing protease inhibitors (complete EDTA-free cocktail tablets, Roche). In a second set of experiments, brushed TEC and cultured TSMC were homogenized in 10 mM Tris·HCl, pH 7.4, 0.4% Triton X-100 containing protease inhibitors (6). Lysates were incubated on ice for 30 min and centrifuged at 14,000 g for 15 min at 4°C. Supernatants were immediately prepared as aliquots and stored at −80°C. Protein concentration in each sample was determined using the Bradford assay (Bio-Rad, Hercules, CA). Arginase activity was assessed using the Quantichrom Arginase assay kit (BioAssay Systems) following the manufacturer’s instructions. Briefly, protein extracts were diluted in double-distilled H₂O to generate an enzymatic activity between 1 and 40 U/l. Protein samples (40 μl) were incubated with L-arginine and manganese-containing buffer for 2 h at 37°C. In some experiments, different end-point times (15 min to 4 h) or L-arginine concentrations (0 to 45 mM final concentration) were evaluated. In the latter, pools of proteins prepared by mixing equal amounts of structural airway tree proteins from each rat (n = 8 rats/group) were used. Rat liver extract served as a positive control. Arginase activity is expressed as units per gram of protein (U/g). One unit of arginase converts 1 μmol of L-arginine to ornithine and urea per minute at pH 9.5 and 37°C.

TSMC proliferation. Rat TSMC from Fischer and Lewis rats were cultured as described above. Cells were used at passages 2 and 3. Fifty percent of confluent cells were starved in a 1:1 Dulbecco’s modified Eagle’s medium/F-12 medium supplemented with 0.1% FBS for 24 h. Cells were pretreated for 30 min with 1 mM nor-NOHA or its corresponding vehicle (DMSO 0.1%) and then incubated with 0.5% or 10% FBS. Proliferation was assayed using bromodeoxyuridine (BrdU) uptake (FITC BrdU Flow Kit, BD Pharmingen). Six hours after the FBS stimulation, BrdU was added to the medium, and smooth muscle cells were collected 18 h later. Results were expressed in percentage of BrdU-stained cells assessed by flow cytometry (FACSCalibur, BD Biosciences).

Statistical analysis. Statistical analysis was performed with GraphPad Prism (GraphPad Software, San Diego, CA). Results were expressed as means and standard errors of the mean (SEM). Physiological parameters were compared with a two-way ANOVA, followed by a Bonferroni post hoc test to compare mean responses at each dose of MCh. Differences observed in gene expression and arginase activity
between the two strains of rat were compared with a Student t-test. P < 0.05 was considered significant.

**RESULTS**

**Measurement of airway responsiveness in vivo.** Fisher rats exhibited higher airway responsiveness to MCh than Lewis rats. The Rs and the Ers were significantly higher in Fischer rat within the range of 16–128 and 4–128 mg/ml of MCh, respectively (P < 0.05, n = 6, Fig. 1, A and B). The analysis by the constant-phase model showed a greater increase in Rn corresponding to the response of the conducting airway, from 4 to 16 mg/ml of MCh (P < 0.05, n = 6, Fig. 1C). Similarly, the Fischer rats exhibited a significantly higher response than the Lewis rats in the parameters reflecting the peripheral tissues, such as G corresponding to the tissue damping and H to the dynamic elastance (Fig. 1, D and E, n = 6, P < 0.05).

**In vivo effect of arginase inhibition on airway responsiveness to MCh.** Treatment with 10 mg/kg of nor-NOHA significantly reduced the MCh-induced increase in Rr and Ers, in both strains of rat (data not shown).

**Total arginase activity, kinetic and progress curves of proximal airway tree.** The arginase activity measured in the structural tissues of the proximal airway tree was similar in Fischer and Lewis rats (Fig. 3A). The Michaelis-Menten plot showed similar enzyme kinetics in the two strains of rat. The Km and Vmax of arginase were 4.3 ± 0.4 mM and 2.9 ± 0.1 U·g⁻¹·min⁻¹, respectively, for the Fischer rats and 4.4 ± 0.5 mM and 3.1 ± 0.1 U·g⁻¹·min⁻¹, respectively, for the Lewis rats. However, arginase extracted from the Lewis rats exhibited a significantly higher activity after 240 min of incubation compared with the Fischer rats (2.5 ± 0.1 vs. 3.3 ± 0.2 U/g, n = 8/group, P < 0.05). At this time point, arginase activity of Fischer reached saturation, while the Lewis enzymatic progress curve was still rising linearly (Fig. 3C).

**Arginase activity and isoform expression in cultured TSMC and in TECs.** The total arginase activity was greater in the TSMC from Lewis rats (10.6 ± 0.2 vs. 6.3 ± 0.8 U/g, P < 0.05, n = 3/group, Fig. 4A). Similarly, the arginase-1 (ARG1) transcript was 3.5 times more expressed in cultured TSMC from Lewis rats compared with Fischer rats (P < 0.05, n = 3/group, Fig. 4B), while arginase-2 (ARG2) was 6.3 times more expressed in Fischer than Lewis. Nitric oxide synthase 3 (NOS3) mRNA expression was not detected in smooth muscle cells (SMC) of either strain of rats. The tracheal epithelial...
arginase activity was detected only in one of the three samples of each group of rats and was close to the detection limit (data not shown). However, mRNA expression of ARG1 was 1.4 times greater in Lewis than Fischer rats \((P < 0.05, n = 3/\text{group}, \text{Fig. 4C})\), while ARG2 mRNA did not differ. NOS3 was 3.8 times more expressed in tracheal epithelium of the Lewis rats compared with the Fischer rats \((P < 0.05, n = 3/\text{group}, \text{Fig. 4C})\).

**Proliferation of the TSMC.** Cultured tracheal smooth muscle from the Fischer rats exhibited a higher proliferation rate than the cells from the Lewis rats in response to 10% FBS \((60.3 \pm 8.1 \text{ vs. } 33.4 \pm 5.2\% \text{ of BrdU-positive cells, } n = 6, P < 0.05)\) or in response to 0.5% FBS \((22.6 \pm 4.9 \text{ vs. } 10.3 \pm 1.5\% \text{ of BrdU-positive cells, } n = 6, P < 0.05)\). One millimole of nor-NOHA inhibition did not influence any of these differences (Fig. 5).

**DISCUSSION**

In this study, we demonstrated that the inhibition of arginase reduced the MCh-induced bronchoconstriction in the large airways of the normoresponsive Lewis rats, while it did not influence the innate AHR of the Fischer rats. While similar kinetics, the arginase activity had a greater saturation capacity in the Lewis rats. Additionally, the pattern of arginase expression was markedly different in the Fischer and the Lewis rats with regard to the cells that composed the airway tree. The structural cells of the Lewis airway tree exhibited a higher arginase expression and activity. There was an associated greater constitutive NOS expression in the Lewis airway epithelium. Although differences in the rates of proliferation of the TSMC of the two strains were observed, these differences were not arginase dependent in either strain of rat.

We confirmed the exaggerated responses of Fischer rats to inhaled aerosols of MCh, as previously reported (8, 19). Additionally, for the first time, we described that the Fischer innate hyperresponsiveness affects all of the compartments of the lung, large and small airways, as well as parenchymal properties, as reflected by the constant-phase parameters RN, H, and G. The hyperresponsiveness observed in the Fischer rats compared with the Lewis rats contrasts with the induced AHR that follows repeated allergen challenges of sensitized Brown-Norway rats where the site of AHR is in the peripheral lung (26). Previous studies examining the role of arginase in the development of AHR in an allergen-driven model of asthma have demonstrated that arginase is involved in the acquisition of AHR associated with allergic airway inflammation (17, 18). In the present rodent model, the absence of airway inflammation allows us to address the role of arginase expressed by the structural tissues of the airways, such as epithelial cells and smooth muscle cells, the effector of the bronchoconstriction. The ability of the two strains to metabolize arginine through either arginase or NOS pathways is anticipated to affect the airway responsiveness to inhaled aerosols of MCh. We evaluated the physiological consequence of an in vivo inhibition of arginase on the innate responsiveness to MCh in the Fischer and the Lewis rats. nor-NOHA did not reduce the airway responsiveness of the Fischer rats, while it did so in Lewis rats. The inhibition was detected with Rrs, Ers, and also RN. The change reflected by the combination of these three parameters localizes the changes to the conducting airways and is sufficiently peripheral that ventilation inhomogeneity may occur to explain the changes in Ers.

To explain the interstrain differences, we explored the importance of arginase in the structural tissues of the proximal tree in several steps. First, we addressed the arginase capacity to metabolize \(\text{l-arginine}\) in the two strains. We found that the overall arginase activity and kinetics were similar in the proximal airway tissue in both strains of rats. However, in the samples from Fischer rats, the arginase achieved saturation more quickly than in the Lewis rats that still showed activity after 240 min. This finding suggests that the Lewis rats have a greater capacity for a sustained metabolism of \(\text{l-arginine}\) by arginase in the proximal airway tree. Second, we addressed the cell types responsible for the observed difference and focused on the structural cells that may have most importance for airway function, namely epithelium and smooth muscle by measuring the arginase activity and the mRNA expression of the two isoforms ARG1 and ARG2. We found a higher arginase activity in the cultured smooth muscle cells compared with the epithelial cells for both strain of rats. The ASM cell arginase activity was higher in the Lewis rats and was associated with a higher expression of ARG1. ARG2 expression was proportionally higher in the Fischer rats. In contrast, NOS3 expression was mainly found in association with the epithelium and was much greater in the Lewis rats, suggesting a greater potential for NO production. This observation is consistent with the previous findings that the airway responsiveness of the Fischer rat was resistant to the effects of the NO donor, sodium nitroprusside, and the inhibitor \(\text{l-NAME}\), whereas the Lewis rat demonstrated a reduction of airway responsiveness following administration of sodium nitroprusside and an increased responsiveness in the presence of \(\text{l-NAME}\). We propose that the inhibition of arginase may have increased the available \(\text{l-arginine}\) for NO synthesis that functionally antagonized the effects of MCh in the Lewis rats through the stimulation of cyclic-guanosine monophosphate synthesis. nor-NOHA has been shown to reduce the response of perfused guinea pig tracheal rings to MCh, and this effect is prevented by coinoculation with a NOS inhibitor, indicating that arginase decreases the NOS-derived NO production (20).
Finally, we tested the possibility that the differences observed in NOS3 and arginase expression in the SMC would explain the higher smooth muscle cell proliferation rate ex vivo in the Fischer rats. The low NOS3 expression in the Fischer epithelium might be expected to favor metabolism through the arginase pathway and would, consequently, lead to the enhanced release of proliferative products derived from arginase and ornithine decarboxylase activity, such as spermidine and spermine. However, the inhibition of arginase did not modify the SMC proliferation in either of the rat strains, indicating that the arginase metabolites produced by the SMC do not directly influence their own proliferation. We cannot exclude the effects of arginase metabolites produced by other pulmonary cells on the ASM cells in vivo. Interestingly, it has been recently demonstrated that arginase inhibition prevents the smooth muscle hyperplasia induced by chronic allergen challenge in the guinea pig (17). Studies addressing the role of arginase in asthma have reported an upregulation of arginase during the early and late asthmatic reaction (15, 17, 18, 23, 24). Arginase inhibitors have been used to demonstrate the role of arginase in airway hyperresponsiveness and smooth muscle remodeling during allergen challenge. In addition to the change in NO synthesis, polyamines such as putrescine and spermine are involved (17, 24). The Fischer-Lewis model of innate AHR, lacking inflammation, suggests a role for arginase in the modulation of airway responses.

In conclusion, the different arginase profiles of expression and activity in the smooth muscle contribute to the differences in sensitivity of the MCh-challenged airways of Lewis and Fischer rats to inhibition of arginase. The innate AHR of Fischer rats is not attributable to arginase, and this strain appears to be resistant to the effects of inhibiting arginase. Likewise, differences in ASM proliferation between the two strains seem unlikely to be caused by differences in arginase expression. However, since the lung arginase activity and isoform expression vary during the fetal life (2), a different expression may influence the lung development and the airway responsiveness earlier in life than what we have studied.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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25. North ML, Khanna N, Marsden PA, Graesemann H, Scott JA. Functionally important role for arginase 1 in the airway hyperrespon-