Enhanced nitric oxide production associated with airway hyporesponsiveness in the absence of IL-10

Bill T. Ameredes, Jigme M. Sethi, He-Liang Liu, Augustine M. K. Choi, and William J. Calhoun. Enhanced nitric oxide production associated with airway hyporesponsiveness in the absence of IL-10. Am J Physiol Lung Cell Mol Physiol 288:L868–L873, 2005. First published December 23, 2004; doi:10.1152/ajplung.00207.2004.—Interleukin (IL)-10 is an anti-inflammatory cytokine implicated in the regulation of airway inflammation in asthma. Among other activities, IL-10 suppresses production of nitric oxide (NO); consequently, its absence may permit increased NO production, which can affect airway smooth muscle contractility. Therefore, we investigated airway reactivity (AR) in response to methacholine (MCh) in IL-10 knockout (−/−) mice compared with wild-type C57BL/6 (C57) mice, in which airway NO production was measured as exhaled NO (ENO), and NO production was altered with administration of either NO synthase (NOS)-specific inhibitors or recombinant murine IL-10. AR, measured as enhanced pause in vivo, and tracheal ring tension in vitro were lower in IL-10−/− mice by 25–50%, which was associated with elevated ENO levels (13 vs. 7 ppb). Administration of NOS inhibitors L-NAME (3 mg/kg ip) or 6-(1-iminoethyl)-lysine (3 mg/kg ip) to IL-10−/− mice decreased ENO, by an average of 50%, which was associated with increased AR, to levels similar to C57 mice. ENO in IL-10−/− mice decreased in a dose-dependent fashion in response to administered rmIL-10, to levels similar to C57 mice (7 ppb), which was associated with a 30% increment in AR. Thus increased NO production in the absence of IL-10, decreased AR, which was reversed with inhibition of NO, either by inhibition of NOS, or with reconstitution of IL-10. These findings suggest that airway NO production can modulate airway smooth muscle contractility, resulting in airway hyporesponsiveness when IL-10 is absent.

airway smooth muscle; exhaled nitric oxide; interleukin-10 knockout mice; methacholine

AIRWAY REACTIVITY (AR) to methacholine (MCh) or other cholinergic agents is utilized as a measure of relative contractility of airway smooth muscle in both humans and animals (6, 19). Assessments of contractility in response to cholinergic stimulation include measurements of FEV1 in response to MCh in humans (24) and measurement of enhanced pause (Penh), airway resistance, and airway smooth muscle tension development in response to MCh in mice (19). These measures are affected by the physiological state of the subject and are used to support clinical diagnoses in humans and differentiation of experimental treatments in animals. For example, MCh challenge in humans can establish the presence of elevated AR associated with asthma (10). Likewise, in animal models, MCh administration can establish links between experimental manipulations of airway inflammation and the consequent airway smooth muscle responses. Furthermore, a reduction in AR to MCh can be indicative of the effectiveness of an exogenous experimental or clinical treatment that reduces airway smooth muscle contractility (1) or the induction of an endogenous agent that reduces contractility (5).

Interestingly, some prior studies have demonstrated reduced AR in mice in which interleukin (IL)-10 was missing (17). Conversely, addition of IL-10 has been observed to increase AR in both mouse and rabbit airway smooth muscle (12, 23, 31). In preliminary studies, we have observed reduced AR in conjunction with increased nitrite levels within bronchoalveolar lavage fluid in IL-10 knockout (IL-10−/−) mice (B. T. Ameredes, unpublished observations). These findings suggested that the absence of IL-10 was associated with reduced airway smooth muscle contractility, perhaps as a consequence of enhanced production of nitric oxide (NO) as previously observed in airway cells from IL-10−/− mice in vitro (2). Furthermore, the observations are consistent with prior data showing suppression of nitric oxide synthase (NOS) expression and activity, and consequently reduced NO production, by IL-10 (7, 25).

Accordingly, we studied AR in IL-10−/− mice and compared them with other strains of mice in which IL-10 was present to determine the link between airway NO production and AR in the presence and absence of IL-10. We hypothesized that a lack of IL-10 would enhance production of NO within the airway and consequently reduce airway smooth muscle contractility. Airway NO production was measured as exhaled NO (ENO) production in vivo in mice with and without administration of NO inhibitors. AR to MCh was measured both indirectly as Penh and directly as the response of tracheal rings in vitro. The results are consistent with the postulate that a lack of IL-10 results in endogenous NO production that decreases airway smooth muscle contractility.

METHODS

General. Male mice of the C57BL/6 (C57, wild-type), IL-10−/− strain (on a C57BL/6 background) were obtained at 6 wk of age (Jackson Laboratories) and housed under identical conditions in a specific pathogen-free (SPF)/barrier animal facility at the University of Pittsburgh. The IL-10−/− mice were certified as double-knockout IL-10 null-mutants (IL-10−/−) originating from a strain produced by Kuhn et al. (18). All mice were allowed to age to 8–12 wk and were subjected to a series of experimental protocols described below. This
maturity window allowed the mice to grow to a sufficient size (18–25 g) for experimental manipulation. Because IL-10−/− mice can develop enterocolitis with age (18), they were routinely monitored for evidence of rectal prolapse and failure to gain weight throughout the study. Although housing under SPF conditions significantly attenuates this tendency (18), mice demonstrating these symptoms were excluded from analysis. All procedures and protocols used in these studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee, which conforms to guidelines recommended by the National Institutes of Health and the United States Department of Agriculture.

In some experiments, mice were injected with either Nω-nitro-L-arginine methyl ester (L-NAME, 8 mg/kg ip, Ref. 3) or L-N(1-iminoethyl)-lysine (L-NIL, 3 mg/kg ip, Ref. 14), dissolved in normal saline every other day over a 3-day period, to inhibit NO production by all NOS isoforms and by inducible nitric oxide synthase (iNOS), respectively, in vivo. Measurements were made 24 h after the last injection. In other experiments, a subdermal implantable miniosmotic pump (Alzet) was placed in the dorsal cervico-scapular area to deliver recombinant murine (rm)IL-10 (R&D Systems) dissolved in a saline vehicle to achieve doses of 25–125 μg·kg−1·day−1; a cohort group of sham saline vehicle alone was used as the control in this case. Measurements were made 72 h after pump implantation.

ENO production. ENO production in unanesthetized mice was measured by a modification of the technique of Weicker et al. (32). In brief, mice were placed individually within a closed acrylic chamber with a low volume (∼600 ml, Buxco) and allowed to breathe freely for a period of time during which the fractional CO₂ content of chamber gas attained 5–5.5%. A small sample of the chamber gas (∼85 ml) was aspirated (20 s, 250 ml/min) in to a gas analyzer (Logan 2500; Logan Research, Kent, UK) that measured NO (sensitivity = 0.3 ppb, time to 90% of peak response <0.5 s), using photometric determination of ozone-enhanced chemiluminescence. The analyzer was calibrated with a certified gas mixture (108 ppb NO) on the morning of each trial and checked periodically with sample gases of known NO concentrations.

In vivo AR measurement. AR was measured in unanesthetized mice as previously described (1). In brief, mice were placed within small-volume (∼600 ml) Plexiglas chambers that allowed for free movement. Alterations in chamber pressure, as a function of mouse breath—volume (VOL), were measured using a modification of the technique of Weicker et al. (32). In brief, mice were placed within small-volume chambers via a DeVilbiss ultrasonic nebulizer connected to an aerosol driver and pump apparatus (Buxco). The administration duration of each MCh concentration was 2 min, followed by a 3-min observation and continued data collection period. The measured response was taken as the highest Penh value achieved during the administration and observation periods.

In vitro AR measurement. Mouse tracheal rings (four to six cartilaginous rings and associated airway smooth muscle; mean wt = 1.7 ± 0.1 and 1.6 ± 0.1 mg for C57 and IL-10−/− mice, respectively) from freshly killed mice (rapid cervical dislocation under Halothane anesthesia; 2% vol/vol, 2 l/min airflow, 5 min) were surgically removed, attached to a wire holder through the lumen of the ring, placed in a 37°C bath (Radnoti) with oxygenated (95% O₂/5% CO₂) Krebs-Henseleit buffer (in mM: 118 NaCl, 2.8 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, and 11.1 dextrose), and connected via a 4-0 silk suture to tension transducers that were interfaced with an analog-digital board (100-Hz sampling rate) on personal computer-compatible computer running a digital data collection program (BioBench, National Instruments). Rings were stretched to attain 500–600 mg passive tension, washed with fresh buffer over 30–60 min, and then exposed to increasing concentrations of KCl (20–60 mM) for intervals of 10–15 min/concentration. After reaching maximal tension at the highest KCl concentration, the rings were washed several times with fresh buffer over a 30-min period. A dose-response relationship was obtained with increasing concentrations of MCh (10−12–10−6 M) applied for 5 min/concentration. Afterward, atropine was applied to inhibit cholinergic tone and to establish that the prior tension increases observed were MCh dependent. At the end of the experiment, the rings were removed, blotted, and weighed.

Statistics. All statistical analyses were performed with Sigma Stat (SPSS) on a microcomputer. A one-way ANOVA was utilized to assess variability in ENO between groups of mice at an alpha level of 0.05, followed by post hoc testing by Student-Newman-Keuls test. Pre- and posttreatment changes in ENO due to NO inhibition were assessed with paired t-tests at an alpha level of 0.05. Changes in ENO due to administration of rmIL-10 were evaluated by one-way ANOVA, followed by application of Dunn’s post hoc test, using the sham saline group as control. A repeated-measures ANOVA was utilized to assess variability in both Penh and tension measurements across MCh concentrations and between groups. With acquisition of a significant F statistic (P < 0.05) by repeated-measures ANOVA, post hoc discreet data analyses between groups within MCh concentrations were performed by Student-Newman-Keuls test, with values of P < 0.05 considered significant.

RESULTS

AR and baseline ENO. In vivo AR was lower in IL-10−/− mice compared with C57 wild-type controls by up to 37% (Penh with 50 mg/ml MCh = 4.4 vs. 2.9, P < 0.05 (Fig. 1). Both strains demonstrated significant AR responses at concentrations of MCh >10 mg/ml, with the IL-10−/− being lower, or right-shifted. ENO levels were significantly greater in naïve IL-10−/− mice (mean = 12.9 ppb, P < 0.05) compared with wild-type mice (6.7 ppb, Fig. 2).

ENO with inhibition of NOS and IL-10 administration. With inhibition of NOS, ENO was significantly decreased in IL-10−/− mice. Inhibition of NOS using L-NAME resulted in decreased ENO in every individual tested (Fig. 3A), producing an average drop of 30% (12.3 to 9.0 ppb, P < 0.05). Inhibition of NOS with L-NIL likewise resulted in decreased ENO in each individual mouse tested (Fig. 3B), producing an average drop of 68% (9.4 to 2.9 ppb, P < 0.05). With administration of rmIL-10 to IL-10−/− mice, ENO was decreased in a dose-dependent fashion compared with sham saline controls (22.3 ppb), with significant decreases observed at doses of 75 (12.1 ppb, P < 0.05) and 125 (6.9 ppb, P < 0.05) μg·kg−1·day−1 (Fig. 4).

Fig. 1. Airway reactivity [as enhanced pause (Penh)] to inhaled methacholine (MCh) in C57BL/6 (C57) wild-type (●) and IL knockout (IL-10−/−) mice (○). ***P < 0.05 vs. respective control (0 MCh) and next lowest MCh concentration, **P < 0.05 C57 vs. IL-10−/− at same MCh concentration and as above; n = 51 mice/group. Values are means ± SE.
AR with NOS inhibition. Tension development in response to KCl stimulation was significantly less in tracheal rings from IL-10−/− mice compared with C57 wild-type controls (mean = 681 vs. 939 mg with 60 mM KCl, P < 0.05; Fig. 5A). Rings from both strains of mice demonstrated responsiveness to all concentrations of KCl, with rings from the IL-10−/− mice being hyporesponsive compared with rings from the C57 wild-type mice. With administration of l-NAME, tension development in rings from IL-10−/− mice was significantly increased, to levels similar to rings of C57 wild-type mice (mean = 869 mg with 60 mM KCl, not significant [n.s.]), whereas tension development in C57 wild-type mice given l-NAME was similar to C57 wild-type mice without l-NAME. Tension development in response to MCh was significantly less in tracheal rings from IL-10−/− mice, being hyporesponsive compared with C57 wild-type controls (816 vs. 1,139 mg with 10−10 M MCh, P < 0.05) and was significantly increased with administration of L-NIL (1,030 mg with 10−10 M MCh, n.s.) to levels similar to rings of C57 wild-type mice with and without L-NAME administration (Fig. 5B). Likewise, AR as measured by Penh in vivo was increased with administration of l-NIL in IL-10−/− mice (Fig. 7).

AR with administration of IL-10 and l-NIL. AR, as measured by Penh in vivo, was increased by 36% with administration of rmIL-10 (1.4 to 1.9 with 50 mg/ml MCh, P < 0.05, Fig. 6), suggesting that replacement of IL-10 in IL-10−/− mice resulted in decreased NO that was associated with an increase in AR.

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Fig. 2. Exhaled nitric oxide (ENO) in individual C57 wild-type (●) and IL-10−/− (○) mice. Means shown with SE bars. *P < 0.05 mean ENO IL-10−/− (n = 28) vs. C57 (n = 19).

Fig. 3. Decreases in ENO in IL-10−/− mice with administration of NO synthase inhibitors. A: ENO in individual IL-10−/− mice (n = 12) given Nω-nitro-l-arginine methyl ester (l-NAME). Means shown with SE bars. *P < 0.05 + l-NAME vs. −l-NAME. B: ENO in individual IL-10−/− mice (n = 4) given l-Nω-(1-iminoethyl)-lysine (l-NIL). Means shown with SE bars. *P < 0.05 + l-NIL vs. −l-NIL.

Fig. 4. ENO in IL-10−/− mice with administration of IL-10. Doses refer to amounts administered through subdermal miniosmotic pump. *P < 0.05 dose 75 (n = 3) and dose 125 (n = 6) vs. sham saline control (0 μg·kg−1·day−1 IL-10, n = 8). Dose 25 (n = 4) was not significant. Bars are means ± SE.

Fig. 5. Airway tension development in tracheal rings from C57 wild-type mice (triangles), IL-10−/− mice (circles), +l-NAME (open symbols); n = 8 mice/group. Values are means ± SE. A: airway tension development in response to increasing concentrations of potassium chloride (KCl). *P < 0.05 vs. respective 0 stimulus concentration, vP < 0.05 IL-10−/− −l-NAME decreased vs. C57 −l-NAME at same concentration, +P < 0.05 vs. IL-10−/− −l-NAME. B: airway tension development in response to increasing concentrations of MCh (statistical symbols as in A).

L870 NO AND HYPORESPONSIVENESS IN ABSENCE OF IL-10

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DISCUSSION

The major findings of this study were 1) a lower level of AR in IL-10^{-/-} mice compared with IL-10-sufficient C57 wild-type mice, 2) a higher level of ENO in IL-10^{-/-} mice compared with C57 wild-type mice, 3) decreased ENO in IL-10^{-/-} mice with inhibition of NOS, 4) increased AR in IL-10^{-/-} mice with inhibition of NOS, 5) decreased ENO with administration of IL-10 to IL-10^{-/-} mice, and 6) increased AR with administration of IL-10 to IL-10^{-/-} mice. These results indicate that a lack of IL-10 is associated with increased airway NO levels, which may predispose the airway toward hyporesponsiveness to cholinergic stimulants. This hyporesponsiveness can be mitigated either with inhibition of NO production using selective inhibitors of NOS activity or through administration of IL-10.

Relationship of NO production and IL-10. The presence of elevated levels of NO within the airways of IL-10^{-/-} mice, compared with IL-10-sufficient mice, is consistent with expectations based on the ability of IL-10 to suppress NOS expression (7); e.g., a lack of IL-10 would represent the loss of a natural brake on NOS activity, with consequent increased NO production (Fig. 2). This initial expected difference was verified by our exhaled gas measurement technique and subsequently was shown to be altered with administration of NOS-specific inhibitors L-NAMe and L-NIL (Fig. 3), similar to findings reported in humans given L-NAMe (29). That the brake on NO production was likely present in the IL-10-sufficient case has been shown previously by Todt et al. (30), who reported measurable IL-10 protein (0.65–0.75 ng/ml) in the lungs of naive C57 mice. Furthermore, reconstitution of IL-10 in IL-10^{-/-} mice resulted in a dose-dependent suppression of airway NO production, down to levels similar to the IL-10-sufficient C57 mice (6–7 ppb, Figs. 2 and 4). These findings strongly suggest that the normally suppressive effect of IL-10 on NO production is apparent as increased airway NO levels in the absence of IL-10.

It might be suspected that iNOS, or NOS II, would predominate production of NO in the absence of IL-10, which could produce increases in ENO and decreased airway resistance (15). However, it is difficult to postulate a strong inductive signal in the absence of stimuli like airway inflammation or the presence of pathogens (3, 20, 25). Our preliminary studies indicated that there is neither measurable difference, nor upregulation of iNOS mRNA or protein, in the lungs of naive C57 wild-type and IL-10^{-/-} mice (data not shown), suggesting that the increases in airway NO are not due to increased levels of this source enzyme in the absence of IL-10. Therefore, the possibility of increased activity of iNOS not associated with transcriptional or translational regulation remains open. The current results displayed a stronger reduction of ENO with L-NIL compared with L-NAMe, which suggests that L-NIL was more effective in suppressing airway NO production, perhaps by iNOS, at the dose level that we used. However, in the absence of an applied experimental stimulus such as airway inflammation, it is also possible that the constitutive/neuronal form, nNOS or NOS I, may dominate (9); furthermore some reports suggest that AR in mice may be linked to the endothelial isoform, eNOS or NOS III (11). These findings indicate that further studies are necessary to determine the relative effective doses of NOS isoform-specific inhibitors in the absence of IL-10, when regulation of NOS may be altered.

Impact on AR. AR was observed to be lower in IL-10^{-/-} mice, compared with wild-type C57 mice, as measured both in vivo and in vitro (Figs. 1 and 5). Subsequent inhibition of NO with NOS-selective inhibitors increased AR (Figs. 5 and 7), suggesting that NO could be acting as a bronchodilator in the case where IL-10 is absent and consistent with prior studies in humans, in which L-NAMe increased AR (29). This interpretation would be consistent with fact that a lack of IL-10 results in increased airway NO production, as observed in the present study. Further support for this notion was found with reconstitution of IL-10 in the IL-10^{-/-} mice, in which airway NO production was significantly reduced, and was associated with an increase in AR (Figs. 4 and 6).

The effects of IL-10, and its lack, on AR have been reported to be variable, ranging from being decreased to being unaltered or increased, and appears to be dependent on the model employed (for review, see Ref. 26). Airway hyporeactivity in the absence of IL-10 has been reported previously, as has the effect of administered IL-10 on restoring AR (12, 17, 23, 27). Current evidence suggests that IL-10 influences or acts in concert with other factors to increase airway smooth muscle activity (17), perhaps involving autocrine signaling through IL-5 (12) or regulation of surfactant protein D (28); however, there has been no further consensus beyond these points. An
additional point to be noted is that many of the models utilize allergen-driven airway inflammation, and most show either no difference or small differences in AR of naïve IL-10−/− mice compared with IL-10-sufficient C57 counterparts. We have further clarified that there can be measurable hyporesponsiveness in naïve IL-10−/− mice, associated with the production of NO, through a mechanism that may involve reductions in airway smooth muscle contractility in response to MCh.

Although we have developed the notion that NO in the absence of IL-10 may be acting as a bronchodilator through actions in airway smooth muscle that may be similar to its actions in vascular smooth muscle (22), another potential explanation for NO-associated airway hyporesponsiveness may actually lie in the action of NO on the vasculature of the lung. For example, in rodent experiments, application of positive end-expiratory pressure (PEEP) is commonly used to return MCh-driven lung resistance levels to precholinergic stimulus levels, in part through induction of pulmonary vasodilation and substrate washout, subsequent to vascular compression by PEEP. It is conceivable that the action of NO to promote pulmonary vasodilation could be bronchoprotective in an indirect fashion that does not necessarily require relaxation of airway smooth muscle. In this case, elevated levels of NO in the lung in the absence of IL-10 could result in NO-induced vasodilatation and enhanced washout that would manifest as airway hyporesponsiveness in the face of a cholinergic challenge, as we observed. This explanation would be consistent with findings that eNOS is a major source of airway NO and is strongly associated with AR in mice (11). Further study is necessary to determine whether this mechanism may be a reason for these observations in this and other models of AR. However, the supporting data from our tracheal ring experiments in vitro argue for a more direct effect of NO on airway smooth muscle function, perhaps as a function of constitutive NOS present in the intact epithelium of those preparations.

Critique of methods. We used the measurement of Penh as an index of AR in vivo, because it allowed a rapid noninvasive assessment of AR measurements in large numbers of mice, using inhaled MCh. However, there has been recent controversy as to whether Penh can accurately reflect changes due to bronchoconstriction in mouse models (21). Although we acknowledge some limitations to the Penh technique, we agree with the assertion by DeLorme and Moss (8), that the choice of the Penh measurement should be linked to the study objective and the ability to track alterations in AR previously verified by more invasive measures of lung resistance. The latter requirement was fulfilled in the present study by assessment of tension development of airway smooth muscle within tracheal rings, in vitro, and by the prior study of Justice et al. (17), in which Penh in C57 and IL-10−/− mice was shown to display results consistent with measures of lung resistance in situ and airway smooth muscle tension development in vitro.

Clinical implications. It has been shown that IL-10 production is decreased, and ENO is elevated, in patients with allergic asthma (4, 16). Those findings are consistent with our present observations of elevated ENO in IL-10−/− mice. However, the alterations in humans occur in asthma with increased AR, a situation that is superficially counterintuitive, as elevated NO production might be expected to decrease AR (15). However, it is possible that elevated ENO in asthma may reflect increased production of NO as a potential homeostatic bronchoprotective agent (29). In this context, enhanced NO could act to minimize AR in the face of strong proinflammatory stimuli and subsequent products that increase AR (12, 28). This may be particularly true in the case of established late-phase allergic asthma, typically characterized by airway inflammation, eosinophilia, elevations in IL-5, and increased AR (5) and could explain the presence of a negative correlation between rising ENO levels and increasing airway hyper-responsiveness (16). In this regard, the notion can be obtained that AR in allergic asthma might be worse were it not for the protective effects of endogenous NO production. Thus it may be that there are powerful feedback mechanisms associated with increased production of NO in the absence of IL-10 that may help to regulate AR. Further study is necessary to elucidate these potential mechanisms and their influence on AR in setting of reduced IL-10 production.

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