

IL-13-induced changes in endogenous glucocorticoid metabolism in the lung regulate the proasthmatic response

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Josephson MB, Jiao J, Xu S, Hu A, Paranjape C, Grunstein JS, Grumbach Y, Nino G, Kreiger PA, McDonough J, Grunstein MM. IL-13-induced changes in endogenous glucocorticoid metabolism in the lung regulate the proasthmatic response. *Am J Physiol Lung Cell Mol Physiol* 303: L382–L390, 2012. First published July 6, 2012; doi:10.1152/ajplung.00125.2012.—Endogenous glucocorticoid (GC) activation is regulated by the intracellular GC-activating and -inactivating enzymes 11 β -hydroxysteroid dehydrogenase (11 β -HSD)1 and 11 β -HSD2, respectively, that catalyze interconversion of inert cortisone and its bioactive metabolite cortisol. Because endogenous GCs are critically implicated in suppressing the asthmatic state, this study examined the roles of the 11 β -HSD enzymes in regulating GC activation and bronchoprotection during proasthmatic stimulation. Airway hyperresponsiveness to methacholine and inflammation were assessed in rabbits following inhalation of the proasthmatic/proinflammatory cytokine IL-13 with and without pretreatment with the 11 β -HSD inhibitor carbenoxolone (CBX). Additionally, IL-13-induced changes in 11 β -HSD isozyme expression and GC metabolism were examined in epithelium-intact and -denuded tracheal segments and peripheral lung tissues. Finally, the effects of pretreatment with CBX or 11 β -HSD2-targeted siRNAs were investigated with respect to cortisol prevention of IL-13-induced airway constrictor hyperresponsiveness and eotaxin-3 production by airway epithelial cells. IL-13-exposed rabbits exhibited airway hyperresponsiveness, inflammation, and elevated bronchoalveolar lung fluid levels of eotaxin-3. These responses were inhibited by pretreatment with CBX, suggesting a permissive proasthmatic role for 11 β -HSD2. Supporting this concept, extended studies demonstrated that 1) IL-13-treated tracheal epithelium and peripheral lung tissues exhibit upregulated 11 β -HSD2 activity, 2) the latter impairs cortisone-induced cortisol accumulation and the ability of administered cortisol to prevent both IL-13-induced heightened airway contractility and eotaxin-3 release from epithelial cells, and 3) these proasthmatic responses are prevented by cortisol administration in the presence of 11 β -HSD2 inhibition. Collectively, these data demonstrate that the proasthmatic effects of IL-13 are enabled by impaired endogenous GC activation in the lung that is attributed to upregulation of 11 β -HSD2 in the pulmonary epithelium.

asthma; cortisone; cortisol; airway hyperresponsiveness; inflammation; airway smooth muscle; epithelium; eotaxin-3

THE ENDOGENOUS GLUCOCORTICOIDS (GCs) regulate a host of critical physiological functions including inflammatory and immune responses, cellular growth and differentiation, and

various metabolic processes (4, 50). These actions at GC-responsive sites are locally regulated by the tissues' ability to modulate the prereceptor bioavailability of GC as a result of intracellular interconversion of the circulating inert GC cortisone and its bioactive metabolite cortisol. Conversion of cortisone to cortisol is catalyzed by the oxoreductase activity of the widely expressed NADPH-dependent GC-activating enzyme, 11 β -hydroxysteroid dehydrogenase-1 (11 β -HSD1), contrasting the opposing GC-inactivating action of the NAD-dependent isozyme 11 β -HSD2, a dehydrogenase that is predominantly expressed in epithelial cells of mineralocorticoid-sensitive tissues (e.g., kidney, colon, lung, etc.), wherein it converts cortisol to cortisone (6, 8–10, 49). As with endogenous GCs, the actions of many synthetic GCs are also regulated by 11 β -HSD-dependent interconversion of their inert and bioactive metabolites (e.g., prednisone and prednisolone interconversion, respectively), while other synthetic GCs (e.g., dexamethasone) are relatively resistant to 11 β -HSD1 but susceptible to inactivation by 11 β -HSD2 (5).

The localized anti-inflammatory actions of GCs are importantly regulated by the immediately surrounding proinflammatory cytokine milieu, which can alter expression of the 11 β -HSD enzymes and, thereby, the intracellular concentration of bioactive GC (6, 49). Accordingly, the Th2-type cytokines IL-4 and IL-13, as well as the pleiotropic cytokines TNF- α and IL-1 β , were shown to induce 11 β -HSD1 expression in human monocytes (46) and vascular and bronchial smooth muscle cells (8, 22), whereas 11 β -HSD2 expression may be down-regulated or upregulated in other cell types (6, 8–10, 13, 22, 54). This suggests that the adverse proinflammatory effects of specific cytokines may either be mitigated or aggravated depending on the nature and degree of induced change in expression of the 11 β -HSD enzymes and their consequent impact on net intracellular GC bioavailability in the affected tissue site. In support of this concept, we recently reported that IL-13-induced upregulation of 11 β -HSD1 and inhibition of 11 β -HSD2 expression in airway smooth muscle (ASM) serve to cooperatively amplify the conversion of cortisone to cortisol by the cytokine-exposed ASM and that the latter phenomenon represents a feedback mechanism that enables physiologically relevant levels of cortisone to exert heightened homeostatic protection of the ASM from the adverse proasthmatic effects of the cytokine on ASM contractility (22). The applicability of the latter phenomenon in regulating the in vivo asthmatic response, however, is open to speculation given that proinflammatory cytokine-induced changes in 11 β -HSD isozyme expression may vary significantly topographically and in different cell types in the

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lung. In light of this consideration, together with evidence demonstrating that endogenous GC release elicited by allergen challenge in asthmatic individuals (7, 14, 37) and in animal models of allergic asthma (11, 15, 31, 40, 42) can significantly affect the intensity of the proasthmatic response, the present study was undertaken to determine the relative contributions of the 11 β -HSD enzymes in regulating the altered airway responsiveness and inflammation elicited by intrapulmonary exposure to IL-13, the key Th2 cytokine implicated in mediating allergic airway disease (17, 52). To our knowledge, our results are the first to identify that IL-13 induces tissue-specific changes in the expression and GC-metabolizing activities of the 11 β -HSD enzymes in ASM, airway epithelium, and peripheral lung tissue and that these differential effects play a crucial role in regulating the overall airway asthmatic response.

MATERIALS AND METHODS

Materials. All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. A549 human airway epithelial cells were obtained from the American Type Culture Collection (Manassas, VA).

Animals. Twenty-four young adult New Zealand White rabbits purchased from Covance were used in this study, which was approved by the Biosafety and Institutional Animal Care and Use Committee of the Children's Hospital of Philadelphia Research Institute, Philadelphia, PA.

IL-13 inhalation and assessment of bronchoconstrictor responsiveness. Rabbits received intraperitoneal injections of either vehicle (saline) or the 11 β -HSD inhibitor carbenoxolone (CBX), administered in a dose of 10 mg/kg in 0.5 ml sterile saline, twice daily times 3 days, as previously reported (43, 46). The following day, the rabbits were challenged with either inhaled vehicle or a maximally effective concentration of IL-13 (20 μ g in 3 ml saline) delivered by nebulization at a flow rate of 8 l/min over ~20 min via a compressor nebulizer (DeVilbiss PulmoMate) connected to an oropharyngeal tube. Twenty-four hours following IL-13 challenge, while mechanically ventilated under general anesthesia/paralysis via an intratracheal cannula connected to a pneumotachograph and pressure transducer, the airflow and transthoracic pressure signals were used to monitor breath-to-breath changes in respiratory system resistance (Rrs) elicited by intravenous bolus injections of cumulatively increasing doses of methacholine (MCh; 0.001–0.15 mg/kg) as previously described (18).

Assessment of pulmonary inflammation. Following determination of MCh responsiveness, the animals were killed with an overdose of pentobarbital sodium (100 mg/kg), the lungs were lavaged in situ with 25 ml of normal saline and then excised and fixed in 10% formalin. Eotaxin-3 levels were assayed in the bronchoalveolar lung fluid (BALF) samples using an ELISA kit (R&D Systems, Minneapolis MN). Paraffin-embedded 4- μ m sections of lung tissue obtained from the lower lobes of both formalin-fixed lungs were stained with hematoxylin and eosin and examined in a blinded manner to assess inflammation.

11 β -HSD1 and -HSD2 protein expression. In separate experiments, tracheae and lungs from naïve rabbits were excised, and peripheral lung tissue sections (each, 1–1.5 cm³) were isolated from the lower lobes. The tracheae were cleaned of loose connective tissue, and the airway epithelium was either left intact or removed by gentle scraping with a cotton-tipped applicator (20, 22). ASM, peripheral lung tissue, and tracheal epithelium samples were minced and homogenized as previously described (20), and the protein concentration was measured using the BCA assay (Thermo Scientific, Rockford, IL). 11 β -HSD1 and -HSD2 protein expression was detected by Western blot analysis using specific primary sheep anti-human targeted antibodies (Binding Site; San Diego, CA). 11 β -HSD expression was also exam-

ined in isolated tracheal and peripheral lung tissues that were transfected over 24 h either with two pooled small interfering RNA (siRNA) duplexes targeted against 11 β -HSD2 (Applied Biosystems: siRNA ID nos. 105991 and 8138) or a nontargeted scrambled RNA (scRNA) sequence serving as a negative control, using Oligofectamine (Invitrogen) as the transfection agent. As previously described (22, 33), each siRNA preparation was administered in a concentration of 100 nM (22, 31) and, based on preliminary studies, this transfection approach was found to greatly enhance transfection efficiency, as evidenced by inhibition of 11 β -HSD2 protein expression by 72–90% in the airway and lung tissue preparations (see RESULTS).

11 β -HSD-dependent cortisone and cortisol metabolism. 11 β -HSD-dependent cortisone and cortisol metabolism were assessed in epithelium-intact and -denuded tracheal preparations and peripheral lung tissues in the absence and presence of IL-13. As previously described (22), following incubation of control and IL-13-stimulated tissues for 24 h in assay buffer containing 1 μ M cortisone or cortisol, together with 500 μ M NADPH or NAD as cofactors, respectively, cortisol levels in the culture medium were assayed by ELISA (R&D Systems). Cortisone metabolism was also determined in tracheal ASM (epithelium intact) and peripheral lung tissues that were initially transfected with either the above 11 β -HSD2 siRNAs or scRNA and, following treatment with cortisone (1 μ M $\times$ 24 h) in the absence and presence of IL-13 (50 ng/ml), cortisol levels in the incubation medium were assayed.

Pharmacodynamic studies of ASM tissue responsiveness. Isolated epithelium-intact tracheal ASM tissue segments exposed overnight to either vehicle or IL-13 (50 ng/ml) under different pretreatment conditions were placed in organ baths containing modified Krebs-Ringer solution and aerated with 5% CO₂ in oxygen at 37°C. As previously described (20, 22, 33), isometric cholinergic contractility to cumulative administration of ACh (10⁻⁹ to 10⁻³ M) was initially assessed and, after rinsing thereafter with fresh buffer, relaxation dose-response curves to isoproterenol (10⁻⁹ to 10⁻⁴ mol/l) were generated in tissues half-maximally contracted with ACh (concentration range: 3 $\times$ 10⁻⁷ to 3 $\times$ 10⁻⁶ M). The constrictor and relaxation dose-response curves were analyzed with respect to each tissue's maximal isometric contractile force (T_{max}) response to ACh and maximal relaxation response (R_{max}) to isoproterenol.

Detection of eotaxin-3 mRNA and protein in human epithelial cells. Human A549 alveolar epithelial cells were grown in Ham's F-12K medium (Lonza; Walkersville, MD) supplemented with 10% FBS and maintained in a humidified incubator containing 5% CO₂ in air at 37°C. After attaining ~85% confluence, cells were starved for 24 h in unsupplemented Ham's F-12K medium, and IL-13-induced temporal changes in eotaxin-3 mRNA expression were determined by RT-PCR, as previously described (22, 33) using the following oligonucleotide primer sets (Integrated DNA Technologies): for eotaxin-3, 5'-AAC-TCCGAAACAATTGTGACTCAGCTG-3' (forward) and 5'-GTA-ACTCTGGGAGGAAACACCCTCTCC-3' (reverse); for β -actin, 5'-GAGAAGAGCTACGAGCTGCCTGAC-3' (forward) and 5'-CGG-AGTACTTGCCTCAGGAGGAG-3' (reverse).

Confluent cultures of A549 cells were also treated for 24 h with vehicle or IL-13 in the absence and presence of either cortisol or CBX alone or in combination, and eotaxin-3 protein released into the culture medium was determined by ELISA (R&D Systems).

Statistical analyses. Results are expressed as means $\pm$ SE or SD values. Comparisons between groups were made using the Student's *t*-test (2-tailed), ANOVA with Tukey posttest analysis, and the non-parametric Kruskal-Wallis test with Dunn's posttest analysis, where appropriate. A *P* of < 0.05 was considered statistically significant. Statistical analyses were conducted by using the Prism computer program by GraphPad Software.

RESULTS

11 β -HSD enzyme activity regulates in vivo proasthmatic responses to IL-13. The net influence of 11 β -HSD enzyme activity in regulating the in vivo pulmonary response to IL-13 was evaluated in rabbits subjected to inhalation of either aerosolized saline or IL-13 (20 μ g) in the absence and presence of pretreatment with vehicle or CBX, an inhibitor of overall 11 β -HSD activity. The following day, bronchoconstrictor responses to MCh were determined as induced changes in Rrs, BALF samples were collected for detection of eotaxin-3 (CCL26) levels, and the lungs were histologically examined for evidence of inflammation. Baseline Rrs values in the control and IL-13-exposed rabbits were not significantly different, averaging 0.028 ± 0.005 and 0.034 ± 0.007 cmH₂O/ml/s, respectively. Relative to controls, however, IL-13-exposed rabbits exhibited marked airway hyperresponsiveness (AHR) to MCh (Fig. 1A), evidenced by pronounced dose-dependent increases in Rrs, wherein the mean $\pm$ SE maximal increase in Rrs (Rrs_{max}) averaged 11.73 ± 1.62 -fold above baseline vs. 3.97 ± 0.97 -fold in the control animals ($P < 0.01$). This AHR was suppressed in IL-13-exposed rabbits that were pretreated with CBX, yielding a mean Rrs_{max} of 6.03 ± 1.33 -fold above baseline, which was not significantly different from that detected in the control animals or in rabbits that were pretreated with CBX alone. Comparably, relative to lungs isolated from control rabbits that showed normal parenchyma and airways with no sign of inflammation, the lungs in IL-13-challenged rabbits exhibited diffusely scattered foci of dense peribronchial, perivascular, and parenchymal inflammatory cell infiltration, predominately with eosinophils and, to a lesser extent, with lymphocytes (Fig. 1B), as well as significantly increased BALF levels of eotaxin-3 (Fig. 1C). These inflam-

matory responses were also suppressed in IL-13-exposed rabbits that were pretreated with CBX.

Airway- and lung tissue-specific effects of IL-13 on 11 β -HSD isozyme expression. Since 11 β -HSD2 prevails in the lung due to its extensive distribution in the epithelial cells lining the large and distal airways and peripheral lung tissue (47), we suspected that the above bronchoprotective action of CBX in the IL-13-exposed animals likely reflected inhibition of the GC-inactivating activity of 11 β -HSD2. This issue was first addressed by comparing constitutive and IL-13-induced changes in 11 β -HSD1 and -HSD2 protein expression in isolated rabbit tracheal ASM and peripheral lung tissues, as well as tracheal epithelium. Immunoblot analysis demonstrated that IL-13-exposed epithelium-denuded rabbit ASM tissues exhibited up-regulated 11 β -HSD1 protein expression (Fig. 2A), whereas constitutively present 11 β -HSD2 was suppressed (Fig. 2B). Induction of 11 β -HSD1 by IL-13 was decreased in ASM tissues that are pretreated with cortisol (Fig. 2A), implicating feedback inhibition of 11 β -HSD1 by the product of its metabolic activity, whereas cortisol pretreatment had no effect on IL-13-induced suppression of 11 β -HSD2 (Fig. 2B). By comparison, peripheral lung tissues displayed low constitutive 11 β -HSD1 expression that was unaffected by IL-13 (Fig. 2A), whereas 11 β -HSD2 was strikingly upregulated by IL-13, and this effect appeared further enhanced by pretreatment with cortisol (Fig. 2B), as previously described in other GC-sensitive cell types (45). Of note, IL-13-induced 11 β -HSD2 expression was markedly inhibited in lung tissues that were transfected with siRNA duplexes directed against 11 β -HSD2 (Fig. 2B), whereas transfection with a scrambled siRNA sequence serving as a negative control had no effect (data not shown), demonstrating the specificity of action of IL-13 on 11 β -HSD2

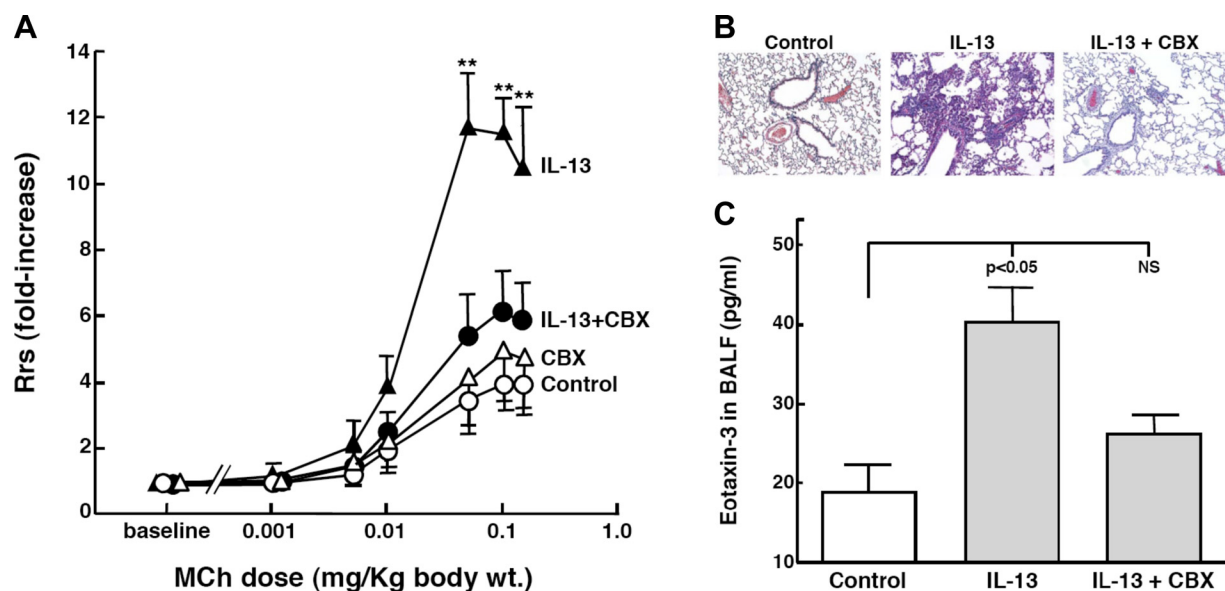


Fig. 1. Pretreatment with the 11 β -hydroxysteroid dehydrogenase (11 β -HSD) inhibitor carbenoxolone (CBX) prevents IL-13-induced in vivo changes in airway responsiveness and pulmonary inflammation in rabbits. A: relative to controls ($n = 4$), methacholine (MCh)-induced increases in respiratory system resistance (Rrs) are significantly enhanced in rabbits at 24 h following inhalation challenge with IL-13 ($n = 4$). This heightened bronchoconstrictor responsiveness to MCh is suppressed in IL-13-exposed rabbits that are treated with CBX prior to inhalation challenge ($n = 4$), whereas bronchoconstrictor responses are unaltered in CBX-treated animals that are not challenged with IL-13 ($n = 3$). Comparably, relative to control rabbit lungs, the lungs in IL-13-challenged rabbits show diffusely scattered foci of dense peribronchial, perivascular, and parenchymal inflammatory cell infiltration (B) and increased bronchoalveolar lung fluid (BALF) levels of eotaxin-3 (C). These pulmonary inflammatory responses are also inhibited in IL-13-exposed rabbits that are pretreated with CBX. Data are means $\pm$ SE values. ANOVA was used for multiple comparisons of mean Rrs values. NS, not significant. $**P < 0.01$.

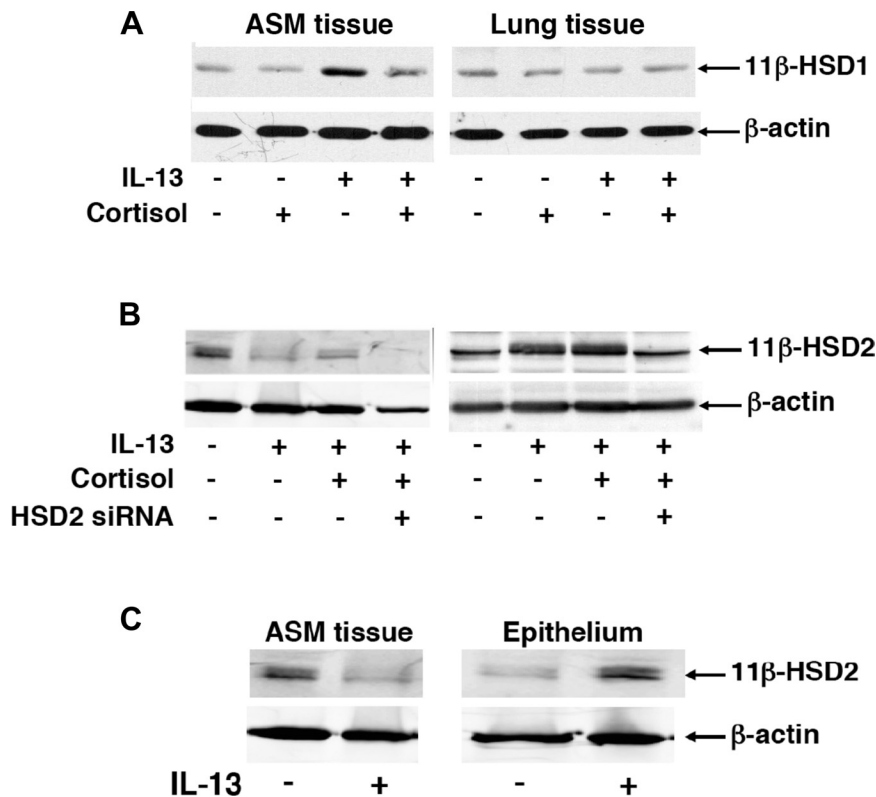


Fig. 2. Western blot analysis comparing IL-13-induced changes in 11 β -HSD protein expression in rabbit airway smooth muscle (ASM) and lung tissues. **A:** IL-13 elicits upregulated expression of 11 β -HSD1 in ASM that is inhibited by pretreatment with cortisol. By comparison, in peripheral lung tissue, 11 β -HSD1 expression is low and unaltered by pretreatment with either IL-13 or cortisol alone or in combination. **B:** IL-13 suppresses basal expression of 11 β -HSD2 in ASM, and this inhibitory effect of IL-13 is unaltered in the presence of cortisol. By comparison, in peripheral lung tissue, 11 β -HSD2 is markedly upregulated by IL-13 and this effect appears enhanced by pretreatment with cortisol. IL-13-induced 11 β -HSD2 expression is suppressed in lung tissues transfected with small interfering RNA (siRNA) duplexes directed against 11 β -HSD2. **C:** in epithelium-denuded tracheal tissue, IL-13 upregulates 11 β -HSD2 in the isolated tracheal epithelium, whereas 11 β -HSD2 expression is suppressed in the adjacent epithelium-denuded ASM.

expression. Finally, as in peripheral lung tissue, IL-13 also upregulated 11 β -HSD2 in isolated tracheal epithelium, whereas, as above, 11 β -HSD2 was suppressed in the adjacent epithelium-denuded ASM (Fig. 2C).

Airway epithelium modulates cortisone and cortisol metabolism: role of 11 β -HSD2 in airway and peripheral lung tissues. Given the observed opposing effects of IL-13 on 11 β -HSD1 and -HSD2 expression in ASM vs. airway epithelium, the role of the epithelium in modulating net airway cortisol bioavailability was examined by comparing the levels of cortisol accumulation in the incubation medium of epithelium-intact vs. -denuded tracheal segments at 24 h following exposure to a physiologically relevant concentration of cortisone (1 μ M), both in the absence and presence of pretreatment with IL-13 (50 ng/ml). Relative to unstimulated controls, IL-13-exposed epithelium-intact tissues exhibited significantly increased cortisol production (Fig. 3A). Correspondingly, in epithelium-denuded tissues, conversion of cortisone to cortisol in unstimulated tissues was increased by approximately twofold above that detected in the unstimulated epithelium-intact controls, and cortisol production was further significantly enhanced by IL-13 to approximately threefold above that in the intact controls. The role of the airway epithelium in regulating cortisol metabolism by 11 β -HSD2 dehydrogenase activity was also examined by comparing the amount of cortisol remaining in the incubation medium of epithelium-intact and -denuded tracheal tissues after 24-h exposure to cortisol (1 μ M; final bath concentration) in the absence and presence of IL-13. Relative to unstimulated controls, IL-13-exposed epithelium-intact tissues exhibited significantly reduced cortisol levels (Fig. 3B), a finding consistent with the observed upregulation of 11 β -HSD2 by IL-13 in the airway epithelium (Fig. 2). By

comparison, this effect of IL-13 was abrogated in epithelium-denuded tissues, wherein cortisol levels were similar to those in unstimulated controls. Taken together, these data support the notion that, whereas airway cortisol bioavailability resulting from 11 β -HSD1 oxoreductase-dependent conversion of cortisone is increased in the presence of IL-13, the net amount of cortisol accumulation in both unstimulated and IL-13-exposed ASM tissues is curtailed by the integrity of the airway epithelium.

The above implied role of 11 β -HSD2 in attenuating cortisol accumulation was further substantiated by comparing the levels of conversion of cortisone to cortisol in unstimulated and IL-13-stimulated isolated control epithelium-intact tracheal and peripheral lung tissues and tissues that were initially transfected with 11 β -HSD2 siRNA duplexes or scRNA. After 24-h exposure to cortisone (1 μ M), relative to controls, IL-13-treated tracheal tissues exhibited significantly increased conversion to cortisol, yielding a mean $\pm$ SE % conversion of 45.9 ± 5.1 vs. $24.1 \pm 2.6\%$ in the controls (Fig. 4A). This heightened cortisol production was similar in IL-13-exposed tissues that were transfected with scRNA, whereas conversion to cortisol was further significantly enhanced in IL-13-treated tracheal segments transfected with 11 β -HSD2 siRNA. By comparison, in peripheral lung tissue, cortisone conversion to cortisol was markedly lower (note different y-axis scales), averaging $4.2 \pm 0.8\%$ under basal conditions (Fig. 4B). Moreover, cortisone conversion to cortisol was further suppressed by IL-13 and, while transfection with scRNA had no effect, cortisol production was significantly increased by IL-13 in tissues transfected with 11 β -HSD2 siRNA. Collectively, these data concur with the above observations in demonstrating that unlike in ASM wherein IL-13 elicits increased expression of

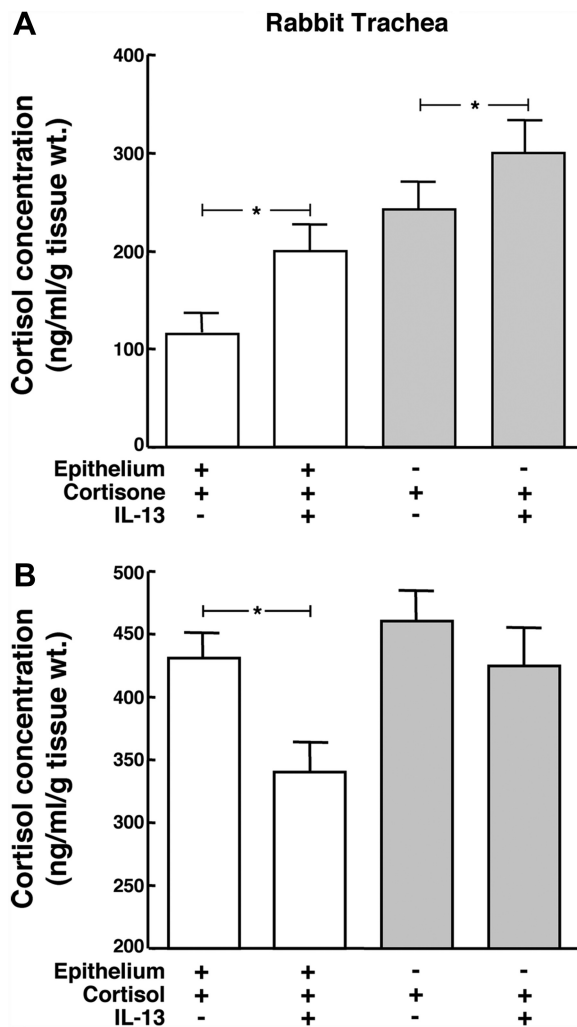


Fig. 3. Comparison of effects of IL-13 on cortisone and cortisol metabolism in isolated epithelium-intact and -denuded rabbit tracheal tissues. **A:** relative to cortisone (1 μ M) conversion to cortisol at 24 h in epithelium-intact controls, cortisol production is significantly increased in epithelium-intact tracheal tissues treated with IL-13. Compared with epithelium-intact controls, cortisone conversion to cortisol is significantly enhanced in unstimulated epithelium-denuded tracheal segments, and cortisol production is further significantly increased in the presence of IL-13. **B:** relative to unstimulated epithelium-intact rabbit tracheal tissues treated for 24 h with cortisol (1 μ M), significantly reduced levels of cortisol are detected in tissues stimulated with IL-13, reflecting IL-13-induced upregulation of 11 β -HSD2 dehydrogenase activity. By comparison, in epithelium-denuded rabbit trachea tissues, similar levels of cortisol are detected in the absence and presence of IL-13 stimulation. Data are means $\pm$ SE values. * P < 0.05.

11 β -HSD1 and suppression of 11 β -HSD2, the latter is upregulated by IL-13 in peripheral lung tissue (as in airway epithelium) (Fig. 2), thereby likely accounting for the reduced cortisol accumulation detected in these preparations and reversal of this phenomenon in the presence of 11 β -HSD2 siRNA.

11 β -HSD2 attenuates the bronchoprotective action of cortisol in ASM. In light of the above results, we tested the hypothesis that IL-13-induced upregulation of 11 β -HSD2 activity in the airway epithelium is permissive of the proasthmatic effects of IL-13 on airway function. Accordingly, the efficacy of cortisol treatment in suppressing IL-13-induced changes in agonist-mediated constrictor and relaxation respon-

siveness was compared in epithelium-intact tracheal ASM segments vs. tissues that were pretreated with CBX or transfected with either 11 β -HSD2 siRNA duplexes or scRNA. As depicted in Fig. 5A, relative to controls, constrictor responses to ACh were significantly increased in tissues that were incubated overnight with IL-13 (50 ng/ml), yielding a mean $\pm$ SE maximal constrictor response ($T_{\max}$) that averaged 139.8 ± 13.2 vs. 113.1 ± 11.1 g/g tissue wt in the controls (P < 0.05). This heightened constrictor responsiveness was unaltered in tissues that were pretreated with cortisol (1 μ M), whereas pretreatment with the same concentration of cortisol abrogated the effect of IL-13 in tissues that were initially exposed to CBX (1 μ M) or transfected with the 11 β -HSD2 siRNA preparation. Comparably, relative to controls, IL-13-exposed tissues also exhibited significantly reduced relaxation responses to isoproterenol (Fig. 5B), yielding a $R_{\max}$ response of 48.8 ± 7.1 vs. $62.3 \pm 6.2\%$ in the controls (P < 0.05). This impaired relaxant responsiveness was also unaltered in tissues pretreated with cortisol, whereas the latter was capable of abrogating the effect of IL-13 in tissues that were initially exposed to CBX or transfected with 11 β -HSD2 siRNA. Of note, results obtained in related experiments demonstrated that pretreatment with cortisol had no significant effect on either 1) the basal $T_{\max}$ or $R_{\max}$ responses to ACh and isoproterenol, respectively, generated in control tissues or tissues that were pretreated with CBX or transfected with scRNA, or 2) the heightened $T_{\max}$ and impaired $R_{\max}$ responses elicited by IL-13 in tissues transfected with scRNA (data not shown).

CBX enables cortisol suppression of IL-13-induced eotaxin production by airway epithelial cells. Since Th2 cytokines are known to evoke GC-sensitive release of eotaxin from cultured airway epithelial cells (1, 25, 28, 29, 32, 44), given the above observations, we next examined whether IL-13-induced release of eotaxin-3 from cultured human A549 alveolar epithelial cells is differentially modulated by cortisol in the absence vs. presence of 11 β -HSD inhibition with CBX. In agreement with previous reports demonstrating cytokine stimulation of eotaxin expression (1, 25, 28, 29, 32, 44), IL-13-exposed A549 cells exhibited temporal increases in eotaxin-3 mRNA expression that peaked at 6 h, with upregulated expression sustained at 24 h (Fig. 6A), and this effect was associated with pronounced eotaxin-3 protein release into the cell culture medium detected at 24 h following IL-13 administration (Fig. 6B). Relative to this response, IL-13-induced eotaxin-3 production appeared somewhat reduced (although not statistically significant) in cells pretreated with CBX (1 μ M) and, while significantly greater inhibition was detected in cells pretreated with cortisol (1 μ M) alone, eotaxin-3 release was markedly suppressed in IL-13-exposed cells that were pretreated with CBX+cortisol. Thus, in concert with the above observations demonstrating a protective action of cortisol when administered in the presence of CBX in IL-13-exposed ASM tissues (Fig. 5), these data demonstrate that the potential for cortisol to suppress IL-13-induced eotaxin-3 production by airway epithelial cells is also enabled by pretreatment with CBX. This evidence is consistent with the in vivo observations demonstrating that together with its bronchoprotective effect, pretreatment with CBX also prevents the pulmonary inflammatory response to IL-13 (Fig. 1).

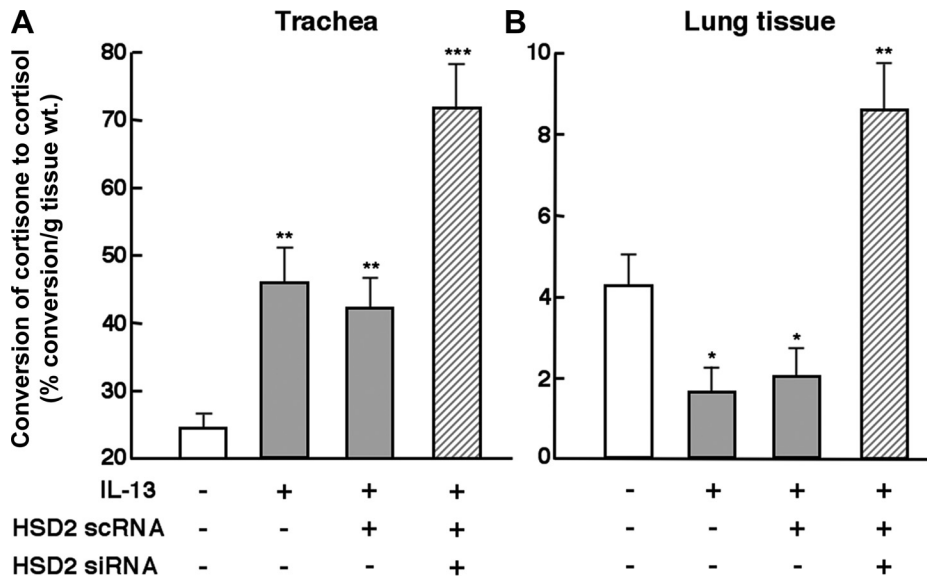


Fig. 4. Comparison of effects of IL-13 on cortisone conversion to cortisol in isolated epithelium-intact rabbit tracheal and peripheral lung tissues. *A*: relative to unstimulated tracheal segments, significantly increased levels of %conversion of cortisone (1 μ M) to cortisol are detected at 24 h in IL-13-stimulated tracheal tissues. *B*: by comparison, compared with unstimulated peripheral lung tissues, significantly reduced levels of %conversion of cortisone (1 μ M) to cortisol are detected at 24 h in IL-13-exposed lung tissues. Note: contrasting the lack of effect of transfection with scrambled RNA (scRNA) (i.e., negative control), cortisone conversion to cortisol is strikingly increased in both IL-13-exposed tracheal and peripheral lung tissues transfected with 11 β -HSD2 siRNA duplexes. Data are means $\pm$ SE values. * P < 0.05; ** P < 0.01; *** P < 0.001.

DISCUSSION

GCs are efficacious in the treatment of asthma because of their ability to suppress both the pulmonary inflammatory response and airway hyperreactivity associated with this disorder. These therapeutic attributes are ascribed to various properties including GC prevention of the release of various proinflammatory cytokines, chemokines, and mediators from stimulated intrapulmonary resident and other cell types, as well as direct GC actions in ASM that result in inhibition of its heightened contractility in the proasthmatic state (19, 21, 26, 33, 36). Like exogenously administered GCs, endogenous GCs have been importantly implicated in inhibiting the asthmatic response (7, 11, 14, 15, 31, 37, 40, 42), and, given that their actions in responsive tissues are critically determined by the intracellular activities of the GC-activating and -inactivating 11 β -HSD enzymes (6, 49), this study sought to identify the roles of these enzymes in regulating the proasthmatic response to IL-13. The results demonstrated that IL-13-induced AHR and pulmonary inflammation in rabbits are suppressed by pretreatment with the 11 β -HSD inhibitor CBX. These *in vivo* observations suggested a permissive role for 11 β -HSD2 in enabling the proasthmatic effects of IL-13, and this notion was supported by observations demonstrating that 1) whereas IL-13 upregulates 11 β -HSD1 and inhibits 11 β -HSD2 expression in ASM, 11 β -HSD2 is upregulated by IL-13 in the airway epithelium and peripheral lung tissue; 2) the latter increase in 11 β -HSD2 and its dehydrogenase activity impairs cortisol bioavailability and, hence, its ability to prevent both IL-13-induced proasthmatic changes in ASM responsiveness and eotaxin-3 release by epithelial cells is curtailed; and 3) inhibition of 11 β -HSD2 activity heightens the bioavailability of cortisol, thereby facilitating its suppression of the proasthmatic response. Collectively, these data provide new insight into the crucial role played by cytokine-induced tissue-specific changes in the expression and GC-metabolizing activities of the 11 β -HSD enzymes in the lung and, accordingly, the bioavailability of endogenous GC to suppress the proasthmatic state.

11 β -HSD1 is expressed in different resident cell types, including ASM cells, wherein its expression is upregulated in

response to the pleiotropic cytokines IL-1 β and TNF- α and the Th2 cytokines IL-4 and IL-13, whereas constitutive 11 β -HSD2 is suppressed (8, 19, 22). Notably, the lung is one of few sites known to also express high levels of 11 β -HSD2, particularly in the epithelia of large and distal airways and peripheral lung tissue (34, 41, 45, 47), suggesting that the bioavailability of cortisol in the lung may be significantly curtailed by the widespread distribution of 11 β -HSD2 dehydrogenase activity (41, 45, 47). In this regard, the present results demonstrated that unlike in ASM, IL-13 elicits upregulated 11 β -HSD2 expression in the airway epithelium and peripheral lung tissue, while having little effect on the relatively modest expression of 11 β -HSD1 in these sites (Fig. 2). Moreover, due to upregulation of 11 β -HSD2 and its dehydrogenase activity, the results demonstrated that 1) net cortisol accumulation derived from oxoreductase-dependent conversion of cortisone is significantly reduced in the IL-13-exposed epithelium-intact airway and peripheral lung tissue, and 2) this inhibitory effect on cortisol bioavailability is abrogated by either removing the airway epithelium (Fig. 3) or pretreating the tissues with 11 β -HSD2 siRNA (Fig. 4). Regarding these observations, it should be noted that the basal levels of conversion of cortisone to cortisol were found to be markedly greater in the trachea vs. peripheral lung tissue. Moreover, unlike in peripheral lung tissue, wherein cortisol production was further attenuated by IL-13 (Fig. 4), the latter increased cortisol production in the tracheal airway, most likely due to IL-13-induced upregulation of 11 β -HSD1 in the underlying ASM (Fig. 2) (22), and this was further enhanced in the epithelium-denuded state (Fig. 3) and in the presence of 11 β -HSD2 siRNA (Fig. 4).

Collectively, the above results raised the hypothesis that due to IL-13-induced upregulation of 11 β -HSD2 in the airway epithelium and lung tissue resulting in reduced bioavailability of cortisol its potential to suppress the proasthmatic effects of the cytokine is impaired. This hypothesis was supported by the results in extended studies demonstrating that, whereas pretreatment of IL-13-exposed ASM tissues with a physiologically relevant concentration of cortisol or with CBX alone had no effect, pretreatment with cortisol+CBX prevented IL-13-

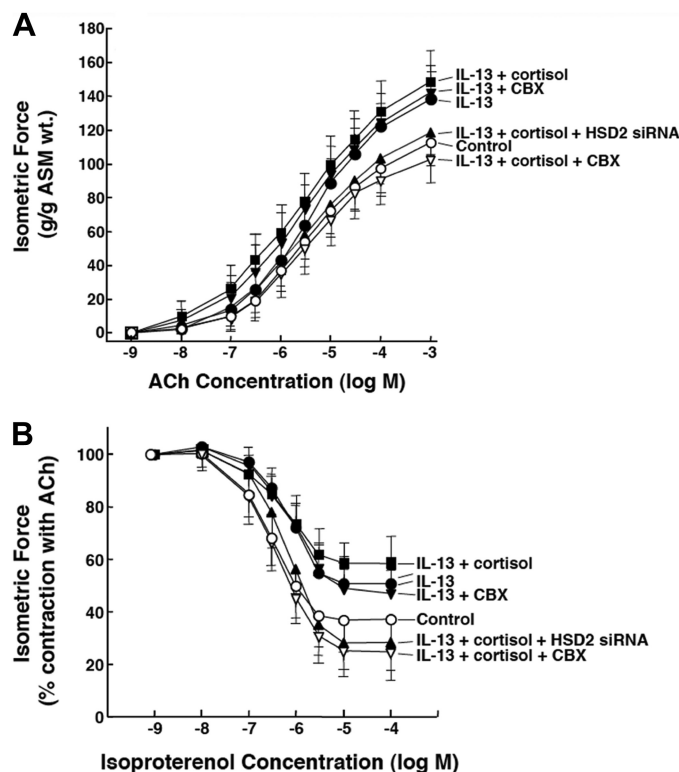


Fig. 5. Inhibition of 11 β -HSD2 activity prevents IL-13-induced changes in ASM constrictor and relaxation responsiveness in isolated epithelium-intact rabbit tracheal tissues. Relative to controls ($\circ$), rabbit ASM tissues exposed for 24 h to IL-13 ($\bullet$) exhibit significantly increased constrictor responses to ACh (A) and impaired relaxation responses to isoproterenol (B). IL-13-induced changes in constrictor and relaxation responsiveness are not significantly altered in tissues pretreated with either cortisol ($\blacksquare$) or CBX alone ($\blacktriangledown$). By comparison, IL-13-induced changes in constrictor and relaxation responsiveness are prevented by cortisol in tissues cotreated with CBX (∇) or transfected with 11 β -HSD2 siRNA duplexes ($\blacktriangle$). Based on ANOVA with Tukey's posttest analysis, for both the dose-response curves to ACh and isoproterenol, differences between control and IL-13-exposed tissues, and between control and IL-13-exposed tissues that are pretreated with either cortisol or CBX alone, are statistically significant ($P < 0.05$). Data are means $\pm$ SD values.

induced changes in ASM constrictor and relaxation responsiveness, similar to the bronchoprotective effect of cortisol in ASM tissues transfected with 11 β -HSD2 siRNA (Fig. 5). Comparably, when evaluating the known suppressive action of GC on Th2 cytokine-induced eotaxin release from airway epithelial cells (28, 29, 44), contrasting the partial (albeit significant) inhibitory effect of cortisol alone, IL-13-induced eotaxin-3 production by A549 epithelial cells was markedly suppressed by cortisol when coadministered with CBX (Fig. 6).

The present observations raise certain relevant considerations. Among these, it is noteworthy that our results largely concur with previous evidence demonstrating that pretreatment with glycyrrhetinic acid, a licorice-derived 11 β -HSD inhibitor similar to CBX, greatly potentiates the ability of hydrocortisone to inhibit IL-13-induced release of GM-CSF from primary cultures of human bronchial epithelial cells, whereas CBX has no effect on inhibition of GM-CSF release by synthetic GCs not metabolized by 11 β -HSD2 or in a transformed human bronchial epithelial cell line that does not express the 11 β -HSD2 enzyme (12). These earlier findings imply that 11 β -HSD2 inhibition alone (i.e., in the absence of

GC) has no inhibitory effect on cytokine-induced GM-CSF release. Our present data demonstrated that although not statistically significant, there was a distinct tendency for treatment with CBX alone to inhibit IL-13-induced eotaxin-3 release from A549 cells (Fig. 6B), an observation consistent with that in an earlier study demonstrating that glycyrrhizin (another 11 β -HSD inhibitor) partially inhibits TNF- α - and IL-4-induced eotaxin-1 production by human fetal lung fibroblasts (27). In this regard, it is important to consider that like glycyrrhizin and glycyrrhetinic acid, CBX was reported to also have intrinsic anti-inflammatory properties (23, 39). Accordingly, these properties may have contributed to our observed tendency for CBX alone to inhibit eotaxin-3 production, and the observed in vivo anti-inflammatory effects of CBX in the IL-13-exposed rabbit lung (Fig. 1). In this connection, it is noteworthy that Ram et al. (38, 39) recently reported that despite inhibition of AHR and pulmonary eosinophilic infiltration elicited by allergen challenge in OVA-sensitized mice, neither glycyrrhizin nor CBX treatment altered serum cortisol

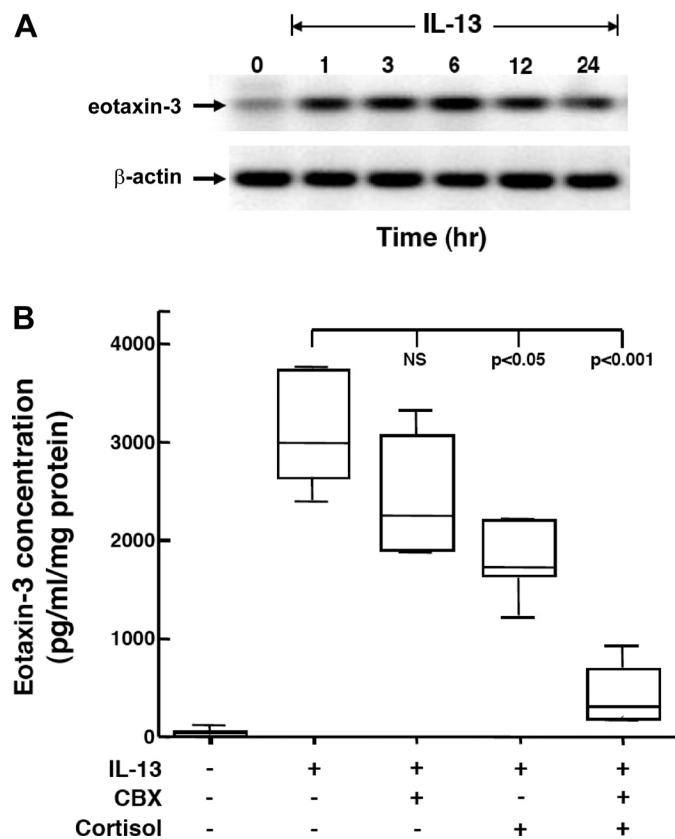


Fig. 6. Carboxinolone facilitates inhibition by cortisol of IL-13-induced eotaxin-3 production by A549 human airway epithelial cells. A: as detected by RT-PCR, IL-13 (50 ng/ml) elicits temporal increases in eotaxin-3 mRNA expression with peak expression detected at 6 h and sustained upregulated expression at 24 h. B: IL-13 evokes pronounced release of eotaxin-3 protein from A549 epithelial cells. Pretreatment with CBX (1 μ M) has no significant inhibitory effect on IL-13-induced eotaxin-3 release and, while significantly greater partial inhibition is obtained in cells pretreated with cortisol (1 μ M), eotaxin-3 release is markedly suppressed in IL-13-exposed cells that are pretreated with the combination of CBX and cortisol. Data are represented by bar plots depicting median and range values. Statistical comparisons are made using the Kruskal-Wallis test, and indicated P values are based on Dunn's posttest analysis comparing results obtained in IL-13-exposed cells to those generated under the different pretreatment conditions.

or BALF corticosterone levels, respectively. Because of this finding, these authors concluded that the bronchoprotective and anti-inflammatory actions of glycyrrhizin and CBX were independent of altered endogenous GC activity (38, 39). Our observations herein, however, do not concur with this conclusion given that unlike when coadministered with cortisol, treatment with CBX alone did not prevent the proasthmatic effects of IL-13 on ASM responsiveness (Fig. 5), and CBX alone had only a modest inhibitory effect on IL-13-induced eotaxin production by A549 cells (Fig. 6). These seemingly opposing conclusions may be reconciled, however, when considering that because the 11 β -HSD enzymes are intracellular and their effects on GC bioavailability are confined to the immediate microenvironment of the responsive tissue (6, 41, 49), a lack of significant change in GC levels detected in serum or BALF is not unexpected. This consideration warrants future systematic investigation.

In evaluating the implications of the present observations, it must be emphasized that our data pertain to *in vivo* studies in rabbits and *in vitro* studies using isolated rabbit airway tissue preparations and cultured human A549 alveolar epithelial cells. Therefore, the extent to which our observations pertain to the human condition is open to speculation. In this regard, it is relevant to note that the endogenous bioactive GC profile in the rabbit is similar to that in humans (i.e., cortisol rather than corticosterone in rodents) and that there exists a greater degree of homology in the amino acid composition of the human and rabbit 11 β -HSD proteins than that between human and rodents (35). Moreover, it should be noted that the changes in constrictor and relaxation responsiveness observed in IL-13-exposed ASM tissues mimicked those previously reported in isolated human asthmatic airways, including enhanced constrictor responsiveness to cholinergic stimulation and impaired β -adrenoreceptor-mediated airway relaxation (2, 3, 16), although there is also evidence that contractility of asthmatic ASM may not be different (51). Finally, regarding our use of A549 cells, a transformed line of alveolar epithelial cells, the issue is raised as to whether our results are reflective of the responses of intrinsic human airway epithelial cells. In this connection, it is important to note that the findings in previously reported studies demonstrate that the human bronchial epithelial cell line BEAS-2B behaves similarly to A549 cells with regard to both cytokine (e.g., IL-1 β , TNF- α , IL-4)-induced eotaxin release (24, 28, 30, 53) and suppression of cytokine-induced eotaxin release by GCs (28, 29). Overall, given these considerations, we believe that the findings described herein are likely applicable to the human condition.

In conclusion, the findings of this study demonstrate that IL-13 elicits topographically distinct tissue-specific changes in the expression and GC-metabolizing activities of the 11 β -HSD enzymes in the lung that importantly impact the overall bioavailability of endogenous GC to suppress the cytokine-induced proasthmatic response. In this context, the findings suggest that future development of interventions specifically targeted at inhibiting 11 β -HSD2 and its GC-inactivating action may offer new approaches to treat asthma.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: M.B.J., J.J., and M.M.G. conception and design of research; M.B.J., J.J., S.X., A.H., C.P., J.S.G., Y.G., G.N., J.M.M., and M.M.G. performed experiments; M.B.J., J.J., P.A.K., J.M.M., and M.M.G. analyzed data; M.B.J., J.J., A.H., and M.M.G. interpreted results of experiments; M.B.J., J.J., A.H., and M.M.G. prepared figures; M.B.J. and M.M.G. drafted manuscript; M.B.J. and M.M.G. edited and revised manuscript; M.B.J., J.J., A.H., C.P., J.S.G., Y.G., G.N., P.A.K., J.M.M., and M.M.G. approved final version of manuscript.

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