Cysteine oxidation impairs systemic glucocorticoid responsiveness in children with difficult-to-treat asthma

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Background: The mechanisms underlying glucocorticoid responsiveness are largely unknown. Although redox regulation of the glucocorticoid receptor (GR) has been reported, it has not been studied in asthmatic patients.

Objective: We characterized systemic cysteine oxidation and its association with inflammatory and clinical features in healthy children and children with difficult-to-treat asthma. We hypothesized that cysteine oxidation would be associated with increased markers of oxidative stress and inflammation, increased features of asthma severity, decreased clinically defined glucocorticoid responsiveness, and impaired GR function.

Methods: PBMCs were collected from healthy children (n = 16) and children with asthma (n = 118) aged 6 to 17 years. Children with difficult-to-treat asthma underwent glucocorticoid responsiveness testing with intramuscular triamcinolone. Cysteine, cystine, and inflammatory chemokines and reactive oxygen species generation were quantified, and expression and activity of the GR were assessed.

Results: Cysteine oxidation was present in children with difficult-to-treat asthma and accompanied by increased reactive oxygen species generation and increased *CCL3* and *CXCL1* mRNA expression. Children with the greatest extent of cysteine oxidation had more features of asthma severity, including poorer symptom control, greater medication use, and less glucocorticoid responsiveness despite inhaled glucocorticoid therapy. Cysteine oxidation also modified the GR protein by decreasing available sulfhydryl groups and decreasing nuclear GR expression and activity.

Conclusions: A highly oxidized cysteine redox state promotes a posttranslational modification of the GR that might inhibit its function. Given that cysteine oxidation is prevalent in children with difficult-to-treat asthma, the cysteine redox state might represent a potential therapeutic target for restoration of glucocorticoid responsiveness in this population. (J Allergy Clin Immunol 2015;136:454-61.)

Key words: *Biomarker, childhood asthma, treatment response, cysteine, inflammation, oxidative stress, refractory asthma, severe asthma*

Glucocorticoids are the cornerstone of treatment for persistent asthma,[1] but the response to these medications is highly variable, particularly when administered at moderate-to-high dosages.[2] Although several large studies have shown that doubling the dose of inhaled glucocorticoids is of limited efficacy in asthmatic patients already receiving low-dose inhaled glucocorticoid therapy,[3,4] other studies suggest that quadrupling the dose might improve measures of asthma impairment and prevent exacerbations.[5] However, chronic inflammation might still persist in a subset of patients with more severe disease,[6] and the mechanisms underlying decreased glucocorticoid responsiveness in this population remain largely unknown.

Although the biology of the glucocorticoid receptor (GR) is complex, posttranslational modifications of the GR and associated downstream effects on glucocorticoid signaling have been described.[7] Although much of the literature has focused on serine phosphorylation, the function of the GR is also regulated by redox-dependent mechanisms,[8,9] which might be of relevance in asthmatic patients. Indeed, we have previously reported marked redox abnormalities in the airways and systemic circulation of asthmatic children that worsen with increased asthma severity.[10-15] Notably, in children with symptomatic asthma despite glucocorticoid therapy, the presence of reactive oxygen species (ROS) promotes oxidation of the amino acid thiol cysteine to its disulfide form (ie, cystine) and further depletes the pool of cysteine available for signal transduction and other cellular functions.[12,13] Given that cysteine oxidation also stimulates cytokine secretion[16] and promotes pathways associated with cellular stress and death,[17] we sought to (1) characterize systemic cysteine oxidation and its intracellular, inflammatory, and clinical features (including glucocorticoid responsiveness) in children with difficult-to-treat asthma and (2) explore the role of cysteine oxidation in GR function. We hypothesized that in children with difficult-to-treat asthma, greater cysteine oxidation would be associated with increased intracellular and extracellular oxidative stress and inflammation, increased features of asthma severity, and decreased glucocorticoid responsiveness. We further hypothesized that cysteine oxidation would impair nuclear GR expression and consensus binding.

METHODS

Participants

Children 6 to 17 years of age with physician-diagnosed asthma who were receiving current asthma treatment were enrolled from a difficult-to-treat asthma clinic at Emory University in Atlanta, Georgia. Asthmatic children had a history of 12% or greater FEV_1 reversibility after short-acting bronchodilator administration or airway hyperresponsiveness to methacholine evidenced by a

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Abbreviations used
ACQ: Asthma Control Questionnaire
E_h: Redox potential
GR: Glucocorticoid receptor
ROS: Reactive oxygen species

methacholine PC_{20} value of 16 mg/mL or less. Healthy children without asthma were also enrolled for comparison. Exclusion criteria for all children included premature birth before 35 weeks' gestation, current smoking, immunodeficiency, pulmonary aspiration disorders, or vocal cord dysfunction.

The Emory University Institutional Review Board granted approval for this study. Written informed consent was obtained from parents or legal guardians. Children 12 to 17 years of age provided written informed assent, whereas children 6 to 11 years of age provided verbal assent.

Participant characterization procedures

Children were evaluated during 2 visits separated by 2 weeks. The baseline characterization visit was rescheduled if upper respiratory tract viral symptoms, acute worsening of asthma symptoms, antibiotic use, or systemic glucocorticoid use was reported within the preceding 2 weeks. Parents completed medical history questionnaires, and children completed the Asthma Control Questionnaire (ACQ) and the Pediatric Asthma Quality of Life Questionnaire.[18,19] Spirometry (KoKo PDS; Ferraris, Louisville, Colo) was performed at baseline and after receipt of up to 8 inhalations of albuterol sulfate (90 μg per actuation). The best of 3 forced vital capacity maneuvers was interpreted.[20] Exhaled nitric oxide concentrations were determined by using online methods (NIOX MINO; Aerocrine, Morrisville, NC).[21] Whole blood (up to 25 mL) obtained by means of venipuncture was collected into serum separation tubes and heparinized tubes containing a density gradient for PBMC isolation (Vacutainer CPT; BD, Franklin Lakes, NJ). Total serum IgE levels were quantified with an assay kit according to the manufacturer's instructions (Calbiotech, Spring Valley, Calif).

Glucocorticoid responsiveness testing

At completion of the baseline visit, a subset (n = 57) of participants with symptomatic asthma despite moderate- to high-dose inhaled glucocorticoid therapy (ie, >200 μg fluticasone equivalent for children 6-11 years of age and >500 μg fluticasone equivalent for children ≥12 years old) received intramuscular triamcinolone (1 mg/kg, 60 mg maximum dose). Symptomatic asthma was defined according to available treatment guidelines[1] as self-reported asthma symptoms more than twice weekly or nocturnal awakenings from asthma at least 2 nights per month. Responsiveness to triamcinolone was assessed after 2 weeks and defined as an ACQ score of less than 0.75, which corresponds to "well-controlled asthma" with a positive predictive value and negative predictive value of 0.73 and 0.85, respectively.[22]

Cysteine and cystine determination

Aliquots were preserved in a 5% perchloric acid solution containing iodoacetic acid (6.7 μmol/L) and boric acid (0.1 mol/L) with 5 μmol/L γ-glutamyl-glutamate internal standard to prevent auto-oxidation before analysis.[23] Cysteine and cystine were quantified relative to γ-glutamyl-glutamate by means of reverse-phase HPLC, as described previously.[12] Samples were derivatized with dansyl chloride and separated on a 10-μm Ultrasil amino acid column (Waters Alliance 2690; Waters, Milford, Mass). Fluorescence was detected at 365 and recorded by 2 using detectors (Waters 474 [Waters] and Gilson 121 [Gilson, Middletown, Wis]). The redox potential (E_h) of the cysteine/cystine thiol pair was calculated with the Nernst equation as follows:

$$E_h = E_o + RT/nF \ln[\text{disulfide}]/([\text{thiol1}][\text{thiol2}]),$$

where E_o is the standard potential for the redox couple (−250 mV), R is the gas constant, T is the absolute temperature, n is the number of electrons transferred, and F is the Faraday constant.

Cellular viability and ROS generation

Cellular viability was determined with an automatic cell counter (Countess; Invitrogen, Grand Island, NY) after staining with 0.4% trypan blue. Intracellular ROS generation was assessed after incubation with 5 μmol/L 2′,7′-dichlorodihydrofluorescin diacetate for 45 minutes. Relative fluorescent units were quantified in 5 to 10 separate visual fields with FluoView software (Olympus, Center Valley, Pa) after correction for background autofluorescence.

RT-PCR

Cells were added to 5 volumes of RNA*later* (Life Technologies, Grand Island, NY), and RNA was extracted with a commercial kit (RNeasy Mini kit; Qiagen, Valencia, Calif). Ten nanograms of total RNA per sample was reverse transcribed with MultiScribe Reverse Transcriptase (62.5 U/50 μL reaction), RNase Inhibitor, oligoT primers, and $MgCl_2$ at a concentration of 5.5 mmol/L (Life Technologies). cDNA aliquots were preamplified for genes of interest by using the TaqMan PreAmp Master Mix Kit (Life Technologies). The preamplified cDNA was used to quantitate relative levels of *CCL3* (Hs00234142_m1) and *CXCL1* (Hs00236937_m1) in a 96-well assay system (StepOnePlus real-time PCR assay) with TaqMan primer pairs and probes (Life Technologies). Data were normalized to *B2M* (4333766F), *GAPDH* (4333764F), *ACTB* (4333762F), *PGK1* (4333765F), and *PPIA* (43337663F) housekeeping genes. Net cycle threshold values were used to calculate Δ cycle threshold values for each subject by using the average of the 5 housekeeping genes as a reference. mRNA gene expression was analyzed relative to the control group and expressed as fold change values.

Cell culture

Cell-culture experiments were performed with THP-1 monocytes (ATCC, Manassas, VA), MM.1S B lymphoblasts (ATCC), and primary PBMCs from healthy donors (AllCells, Alameda, Calif). Reduced and oxidized conditions were created by adding 300 μL of 10 mmol/L cysteine and 150 μL of 10 mmol/L cystine (Sigma-Aldrich, St Louis, Mo), respectively, to 15 mL of Dulbecco modified Eagle medium/F12 media without methionione, cysteine, cystine, and glutamine (HyClone, Life Technologies). Media was supplemented with 10% FBS, 1% penicillin/streptomycin (Cellgro; Corning Life Sciences, Corning, NY), and 50 mg/mL gentamicin sulfate (Cellgro). Cells were resuspended to a concentration of 1 million cells/mL and cultured for 4 hours at 37°C with 5% CO_2. In selected experiments cells were also exposed to 100 nmol/L dexamethasone for 1 hour (Sigma-Aldrich), which was added at hour 3 of incubation.

Western blotting

Cells were suspended in 50 mmol/L Bis-Tris-HCL lysis buffer (pH 6.5) containing 0.5% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 150 mmol/L NaCl, 1 mmol/L EDTA, and 0.1 mmol/L phenylmethylsulfonyl fluoride. Protein sulfhydryl group (-SH) protein residues were labeled with biotinylated iodoacetamide by using the methods of Go et al[24] at a final concentration of 20 μmol/L for 15 minutes, after which iodoacetamide was added to a final concentration of 5 mmol/L. The GR was immuneprecipated with an anti-GR receptor antibody (Santa Cruz Biotechnology, Dallas, Tex) and the Protein G Immunoprecipitation Kit (Sigma-Aldrich). Eluted sample was divided and run on two 10% SDS-PAGE gels. Proteins were transferred overnight to nitrocellulose membranes, and biotinylated iodoacetamine binding was visualized on an Odyssey Classic Infrared Imaging System (LI-COR, Lincoln, Neb) by incubating with streptavidin-conjugated IRDye 680RD (LI-COR) for 1 hour. Even loading of samples was determined by incubating the second membrane overnight at 4°C with human GR antibody raised in rabbit (Santa Cruz Biotechnology). Visualization was performed after secondary incubation with anti-rabbit IgG conjugated to IRDye 680RD (LI-COR).

Nuclear isolation, total protein measurement, and nuclear GR expression and activation

Nuclei were isolated with a commercial nuclear extract kit (Active Motif, Carlsbad, Calif). Protein concentrations were measured at 750 nm with BSA as

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