Correlation of Systemic Superoxide Dismutase Deficiency to Airflow Obstruction in Asthma

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Online Data Supplement

METHODS

Study population

To evaluate SOD in serum, the study population included 135 individuals comprised of 20 healthy nonsmoking individuals and 115 asthmatic individuals (75 non-severe and 40 severe asthmatics). All samples were collected by investigators in the NHLBI Severe Asthma Research program (SARP). Severe asthma was based on the definition used by the proceedings of the American Thoracic Society Workshop on Refractory Asthma (1), with major and minor characteristics. Defining major characteristics include (1) treatment with continuous or near continuous oral corticosteroids, and/or (2) high dose inhaled corticosteroids. The minor criteria are as follow: (1) Daily treatment with controller medication in addition to inhaled corticosteroids; (2) use of short-acting β-agonist on a daily or near daily basis; (3) Persistent airway obstruction [FEV_1>80% predicted and diurnal peak expiratory flow (PEF) variability >20%]; (4) one or more urgent care visits for asthma per year; (5) Three or more oral corticosteroid bursts per year; (6) prompt deterioration with reduction in oral or inhaled corticosteroid dose; (7) Near-fatal asthma event in the past.

Subjects enrolled in SARP were classified as healthy controls, non-severe or severe asthma. Subjects met criteria for severe asthma with at least 1 major and at least 2 minor criteria. Inclusion criteria for control subjects were (1) lack of cardiopulmonary symptoms, (2) normal baseline spirometry, and (3) a negative methacholine challenge test (defined as less than 20% decline in FEV_1 with the maximum dose of methacholine). Exclusion from SARP enrollment for asthmatic and control subjects included current smoking history, or smoking history within one year, former smokers with greater then 5

pack-year total history, pregnancy and human immunodeficiency virus infection. The study was approved by all SARP centers Institutional Review Boards and written informed consent was obtained from all individuals.

Procedures to characterize volunteers

Lung function. Spirometry was performed on an automated spirometer consistent with American Thoracic Society standards. The FVC, FEV_1, and FEV_1 to FVC ratio were collected for each of three efforts before and after the administration of two albuterol puffs via Aerochamber. Reference equations for spirometry are those of National Health and Nutrition Examination Survey (NHANES III).

Atopy. All volunteers underwent skin testing with the Multi-Test II (Lincoln Diagnostics, Inc). Allergy skin testing was performed with the following antigens: cat allergen, dog hair, D. Pteryn, D. Farinae, cockroach, tree mix, ragweed mix, common weed mix, molds including Atlternaria, Aspergillus, and Cladosporium, normal saline as negative control, and histamine as positive control. Allergens were obtained from Hollstier Stier, Spokane, Washington and tested to make sure they are free of lipoplysaccharide (LPS) contamination. Fifteen minutes after the application of the allergen, a study coordinator assessed redness and/or swelling at the site. Significant tests were those in which the application of an allergen produces a wheal with diameter of 3 mm or more than the negative control or a flare with diameter of 10 mm or more. Allergy or atopy was defined as two or more positive skin tests in the presence of positive histamine reaction. Allergy skin testing was done once on each subject during the study.

Airway reactivity. Methacholine challenge testing was performed in all volunteers. However, patients with a baseline %FEV_1 lower than 55% did not undergo a methacholine challenge. The degree of airway narrowing was measured by using the forced expiratory spirometry, particularly FEV_1. Increasing concentrations of methacholine were delivered until FEV_1 fell at least 20% when compared to a control (postdiluent) level. The measure used to compare the sensitivity of one individual to another was PC_{20}, the first provocative concentration that caused a 20% fall in FEV_1.

Extracellular Glutathione Peroxidase protein (eGPx).

eGPx protein was measured by enzyme-linked immunosorbent assay (ELISA) (Calbiochem, La Jolla, CA). This method is based on a sandwich-type immunoassay, and is specific for eGPx. The eGPx protein concentration present in serum was obtained using a 4-parameter curve fit generated from known standard concentrations of human eGPx.

Total GSH (GSH+GSSG).

GSH levels in serum were measured by standard methods as previously described (26). In brief, total glutathione levels were determined by mixing equal volumes of serum with 10 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in 100 mM potassium phosphate, pH 7.5, which contained 17.5 μM EDTA. An aliquot (50μl) of the solution was added to a cuvette containing 0.5 U of glutathione disulfide reductase (Sigma type III, Sigma Chemical, St. Louis, MO) in 100 mM potassium phosphate and 5 mM EDTA, pH 7.5. After 1 minute, the reaction was initiated with 220 nmol of NADPH in a final reaction

volume of 1 ml. The rate of reduction of DTNB was recorded continuously at 412 nm by a spectrophotometer with a Kinetics/Time feature (Beckman DU-640, Beckman Instruments, Inc. Fullerton, CA).

Glutathione Peroxidase (GPx) activity

Total glutathione peroxidase activity was determined spectrophotometrically in serum. Serum was incubated in the presence of 0.1 mM sodium azide, 1 U/ml glutathione reductase, 0.1 mM glutathione and 0.12 mM reduced β-nicotinamide adenine dinucleotide phosphate (β-NADPH), 0.016 mM dithiothreitol, 0.38 mM EDTA and 50 mM sodium phosphate (pH 7.0) for 2 minutes at 25^{o}C. The reaction was initiated by the addition of 0.2 mM hydrogen peroxide. The decrease in absorbance at 340 nm over 3 minutes as NADPH is converted to NADP is proportional to the GPx activity. One unit of activity is defined as the activity that catalyzed the oxidation of 1 nmol NADPH/min using an extinction molar coefficient of 6.22×10^{6} M^{-1} cm^{-1} for NADPH (1).

SOD activity assay.

SOD activity was determined by the rate of reduction of cytochrome c, with one unit (U) of SOD activity defined as the amount of SOD required to inhibit the rate of cytochrome c reduction by 50%(2). The final reaction volume was 3 ml and included 50 mM potassium phosphate buffer, 2 mM cytochrome c, 0.05 mM xanthine, and a 0.1 mM EDTA solution. Xanthine oxidase (Sigma, St Louis, MO) was added at a concentration sufficient to induce a 0.020 per minute change in absorbance at 550 nm.

Sample preparation and mass spectrometry.

Protein-bound 3-nitrotyrosine, 3-bromotyrosine, and o,o'-dityrosine were determined by stable isotope dilution liquid chromatography–tandem mass spectrometry on a triple quadrupole mass spectrometer (Quattro II Ultima, Micromass, Inc.) interfaced to a Cohesive Technologies Aria LX Series HPLC multiplexing system (Franklin, MA) (3, 4). Briefly, aliquots of plasma (200 μg protein) were desalted and delipidated using a single phase extraction mixture comprised of aqueous sample:methanol:water-washed diethyl ether (1:3:7; v/v/v), the protein pellet supplemented with isotope labeled internal standards ([$^{13}C_{9,}{}^{15}N_1$]tyrosine, 3-bromo[$^{13}C_6$]tyrosine, 3-nitro[$^{13}C_6$]tyrosine, and *o,o′* di-[$^{13}C_{12}$]tyrosine for quantification of the respective parent and oxidized amino acids), subjected to acid hydrolysis with methane sulfonic acid, passed over solid-phase C18 extraction columns (Supelclean LC-C18-SPE minicolumn; 3 ml; Supelco, Inc., Bellefone, PA), and then analyzed by injection onto reverse phase analytic HPLC columns interfaced with the mass spectrometer using multiple reaction monitoring mode for characteristic parent/daughter ion transitions for each analyte and its appropriate isotopomers (5). Results are normalized to the content of the precursor amino acid tyrosine, which was monitored within the same injection. Intrapreparative formation of [$^{13}C_9{}^{15}N$]tyrosine-derived 3-bromotyrosine, o,o'-dityrosine and 3-nitrotyrosine were routinely monitored for in all analyses and shown to be negligible under the sample preparation conditions employed (i.e. < 5% of the level of the natural abundance product observed).

Superoxide dismutase treatment with eosinophil peroxidase-derived reactive species.

CuZnSOD (Calbiochem, La Jolla, CA) with specific activity of 3.78 U/μg protein was exposed to the eosinophil peroxidase (120 nM final)/H_20_2 (100 μM final) system in the presence of either sodium bromide (100 μM final), nitrite (100 μM final) or tyrosine (100 μM final) for 30min at 37 °C. Reactions were performed in potassium phosphate buffer (15 mM, pH 7.0) supplemented with 200 μM diethylenetriaminepentaacetic acid. Reactions were quenched by addition of methionine (100 μM) and snap freezing in liquid nitrogen.

Statistical Analysis.

Data were summarized using the mean and its standard error (SEM). Group comparisons were performed with analysis of variance (ANOVA), and tests were performed at individual significance levels of α=0.05 (i.e., $p<0.05$ was considered significant). Associations between SOD activity and each of age, gender, and medication were assessed using linear models and ANOVA, and these factors were included as covariates in linear models for the group comparisons. All tests and model-fitting were performed with the R statistical language, version 1.9.0

(R=Development Core Team (2004). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-00-3, URL http://www.R-project.org.)

REFERENCE

E1. 2000. Proceedings of the ATS Workshop on Refractory Asthma . Current Understanding, Recommendations, and Unanswered Questions. *Am. J. Respir. Crit. Care Med.* 162(6):2341-2351.

E2. Nebot, C., M. Moutet, P. Huet, J. Z. Xu, J. C. Yadan, and J. Chaudiere. 1993. Spectrophotometric assay of superoxide dismutase activity based on the activated autoxidation of a tetracyclic catechol. *Anal Biochem* 214(2):442-51.

E3. Brennan, M. L., W. Wu, X. Fu, Z. Shen, W. Song, H. Frost, C. Vadseth, L. Narine, E. Lenkiewicz, M. T. Borchers, A. J. Lusis, J. J. Lee, N. A. Lee, H. M. Abu-Soud, H. Ischiropoulos, and S. L. Hazen. 2002. A tale of two controversies: defining both the role of peroxidases in nitrotyrosine formation in vivo using eosinophil peroxidase and myeloperoxidase-deficient mice, and the nature of peroxidase-generated reactive nitrogen species. *J Biol Chem* 277(20):17415-27.

E4. Eiserich, J. P., S. Baldus, M. L. Brennan, W. Ma, C. Zhang, A. Tousson, L. Castro, A. J. Lusis, W. M. Nauseef, C. R. White, and B. A. Freeman. 2002. Myeloperoxidase, a leukocyte-derived vascular NO oxidase. *Science* 296(5577):2391-4.

E5. Zheng, L., B. Nukuna, M. L. Brennan, M. Sun, M. Goormastic, M. Settle, D. Schmitt, X. Fu, L. Thomson, P. L. Fox, H. Ischiropoulos, J. D. Smith, M. Kinter, and S. L. Hazen. 2004. Apolipoprotein A-I is a selective target for myeloperoxidase-catalyzed oxidation and functional impairment in subjects with cardiovascular disease. *J Clin Invest* 114(4):529-41.