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Original Article

Malondialdehyde and 3-Nitrotyrosine in Exhaled Breath Condensate in Retired Elderly Coal Miners with Chronic Obstructive Pulmonary Disease

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ABSTRACT

Background: Chronic obstructive pulmonary disease (COPD) is an important cause of occupational mortality in miners exposed to coal mine dust. Although the inflammatory mediators involved in COPD have not been defined, many studies have shown that inflammatory mediators such as reactive oxygen and nitrogen species are involved in orchestrating the complex inflammatory process in COPD.
Methods: To investigate the relevance of exhaled biomarkers of oxidative and nitrosative stress in participants with COPD, we determined the levels of hydrogen peroxide, malondialdehyde (MDA), and 3-nitrotyrosine (3-NT) in exhaled breath condensate (EBC) in 90 retired elderly coal miners (53 non-COPD and 37 COPD participants).
Results: Mean levels of MDA (4.64 nM vs. 6.46 nM, $p = 0.005$) and 3-NT (3.51 nM vs. 5.50 nM, $p = 0.039$) in EBC were significantly higher in participants with COPD. The median level of MDA did show statistical difference among the COPD severities ($p = 0.017$), and the area under the receiver operating characteristic curve for MDA (0.67) for the diagnostic discrimination of COPD indicated the biomarker. The optimal cutoff values were 5.34 nM (64.9% sensitivity and 64.2% specificity) and 5.58 nM (62.2% sensitivity and 62.3% specificity) for MDA and 3-NT, respectively. The results suggest that high levels of MDA and 3-NT in EBC are associated with COPD in retired elderly miners.
Conclusion: These results showed that the elevated levels of EBC MDA and EBC 3-NT in individuals with COPD are biomarkers of oxidative or nitrosative stress.
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1. Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response to noxious particles or gases in the airways and lungs [1]. Among occupational lung diseases, the most prevalent diseases are induced by inhalation of dust, including asbestos, crystalline silica, and coal. Exposure to silica and coal mine dust may result in pulmonary scarring in a pattern that mimics idiopathic pulmonary fibrosis, and in COPD that appears indistinguishable from the obstructive lung disease caused by exposure to tobacco smoke [2]. Coal workers' pneumoconiosis and COPD are important causes of occupational mortality in miners with extensive exposure to coal mine dust [3].

The role of inflammatory mediators in the development of COPD has not been clearly defined, but it is now apparent that many of them, such as reactive oxygen and nitrogen species, cytokines, chemokines, and growth factors, are involved in orchestrating the complex inflammatory process in COPD [4]. One of the pathogenic processes of COPD is oxidative stress, which may be a mechanism that enhances the inflammatory response [5].

Detection of inflammatory biomarkers of pulmonary diseases involves invasive techniques, such as obtaining samples of blood, bronchoalveolar lavage fluid, and induced sputum. Because invasive sampling procedures may induce an inflammatory response, noninvasive techniques, such as sampling exhaled breath condensate (EBC) and exhaled air, have been developed. Analysis of various biomarkers in exhaled breath has allowed noninvasive monitoring

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of inflammatory and oxidative stress in the respiratory tract in inflammatory lung diseases [6–9]. Malondialdehyde (MDA) has been widely studied as a product of polyunsaturated fatty acid peroxidation resulting from oxidative stress. Increased MDA levels have been observed in several biological fluids obtained from patients with different airway diseases including asthma and COPD [10]. Exhaled nitric oxide (NO) released from lung cells, including inflammatory cells, epithelial cells, and endothelial cells in the respiratory tract, has been known to be an effective biomarker for diagnosis and treatment of lung disorders including COPD [11–13]. The main form of NO degradation is the rapid reaction with superoxide anion to form the more reactive product peroxynitrite. Peroxynitrite reacts with tyrosine to form 3-nitrotyrosine (3-NT) [14]. Thus, NO and protein-associated NT are considered as characteristic markers of nitrosative stress [15]. There have been relatively few reports analyzing EBC biomarkers in elderly retired coal miners with COPD.

In this study, we hypothesized that the levels of biomarkers of oxidative and nitrosative stress in the EBC of patients with COPD are elevated. To test this hypothesis, we evaluated the hydrogen peroxide (H_2O_2), MDA, and 3-NT levels in the EBC.

2. Materials and methods

2.1. Participants

A group of 90 retired male miners were recruited and examined for pneumoconiosis at Ansan Workers'z Compensation Hospital, Korea Workers' Compensation and Welfare Service (KCOMWEL). The data collected included the forced vital capacity (FVC) and the forced expiratory volume in 1 second (FEV_1). Personal information, including age, body mass index (BMI), and demographic information such as job and smoking status were obtained using a structured questionnaire. Pulmonary function testing was performed in accordance with international guidelines using a Vmax22 spirometer (SensorMedics, San Diego, CA, USA) [16]. FVC, which is the volume delivered during an expiration made as forcefully and completely as possible starting from full inspiration, and FEV_1 were measured. A clinical diagnosis of COPD was considered in participants who showed an FEV_1/FVC ratio $< 70\%$ and without FEV_1 increasing higher than 200 mL and 12% after β_2-bronchodilator inhalation.

Chest radiographs for the diagnosis of pneumoconiosis were obtained and scored according to the International Labor Office classification rules [17] by an experienced panel of physicians from the Pneumoconiosis Review Committee associated with KCOMWEL.

2.2. EBC collection

Collection of EBC was performed in accordance with the guidelines of the American Thoracic Society [18]. In brief, all participants rinsed their mouths with purified saline prior to collection. Between 1 mL and 3 mL of EBC was collected during 10 minutes of oral tidal breathing using an EBC collection system (ECoscreen 2; FILT GmBH, Berlin, Germany). The participants wore nose clips while collecting the condensate to prevent air from escaping. The collected EBC samples were immediately stored at $-80°C$ and analysis was performed within 1 month of collection.

2.3. Analysis of biomarkers in EBC

The H_2O_2 levels in EBC were measured by automated flow injection analysis with fluorescence detection (high-performance liquid chromatography system; Agilent 1200 Series; Agilent technologies, Waldbronn, Germany), as previously described [19]. In brief, 150 μL of EBC samples or standard was added to 15 μL of a mixture of 2.5 U/L horseradish peroxidase and 2.5 mM *p*-hydroxyphenylacetic acid. The injection volume was 20 μL and the excitation and emission wavelengths were 285 nm and 400 nm, respectively.

Analysis of MDA levels in EBC samples was performed by liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry (LC-APCI-MS/MS, 3200QTRAP, AB SCIEX, CA, USA; Fig. 1), as previously described [20]. In brief, samples were derivatized with 2,4-dinitrophenylhydrazine solution (12 mM in acetonitrile and 2% formic acid) at room temperature for 60 minutes, after which 20 μL of the derivatized samples was injected onto the LC-APCI-MS/MS system (3200QTRAP). Chromatography of derivatives was performed on a C18 reverse-phase column (Triart-C18; 2.0 mm × 100 mm, 3 μm, YMC Co. Ltd, Shimogyo-ku, Kyoto, Japan) using 20 mM aqueous acetic acid and methanol under gradient elution condition at a flow rate of 0.2 mL min^{-1}. The gradient program was as follows: from 45% to 98% methanol in 4 minutes, linear gradient, and then hold for 4.5 minutes. The target ion of derivatives was *m/z* 235/159 in the positive mode.

A sandwich enzyme immunoassay was used to measure 3-NT (NWLSS Nitrosine ELISA Kit, NWK-NTR01, Northwest Life Science Specialties, Vancouver, Canada) levels in accordance with the manufacturer's instructions.

2.4. Statistical analyses

Levels of H_2O_2 and 3-NT in EBC showed a log-normal distribution. The values were log-transformed for parametric statistical tests, and the parametric unpaired Student *t* test and analysis of variance test were used to determine the magnitudes of between-group differences. If the sample sizes among the study groups were also different or the number of cases for each group were incompatible for parametric statistical tests, the nonparametric Mann–Whitney *U* test and Kruskal–Wallis one-way analysis based on rank sums were used to determine differences among the study groups. A multiple regression model was constructed to compare non-COPD participants with those who had COPD, which was then adjusted for age, BMI, exposure period, smoking status, and pneumoconiosis. Receiver operating characteristic (ROC) curve analysis was used to assess the potential of each exhaled biomarker for the discrimination of COPD. Statistical cutoff values were calculated by minimizing the distance between the point with specificity = 1 and sensitivity = 1 and various points on the ROC curve. For ROC analysis, an area under the curve (AUC) of 1.0 indicates perfect discrimination, whereas an area of 0.5 indicates that the test discriminates no better than chance. The relationship between biomarkers and effective variables was evaluated using stepwise multiple regression analysis. Values of $p < 0.05$ were considered statistically significant. All statistical evaluations were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

2.5. Ethics statement

The study protocol was approved by our institute's Research Ethics Committee (Approval number 2010-03-04). All participants signed an informed consent form that conformed to the recommendations of the committee.

3. Results

3.1. General characteristics

The general characteristics of the study participants are shown in Table 1. Of the 90 study participants, 37 were diagnosed with COPD, whereas the remaining 53 were classified as the non-COPD

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