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THE INTERACTIONS OF CARBON BLACK PARTICLES WITH REDUCED GLUTATHIONE AND PROTEINS IN RESPIRATORY TRACT LINING FLUID

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Abstract. In this study we examined the impact of carbon black particles (CBPs) type M120 on the reduced glutathione and proteins in the respiratory tract epithelial lining fluid (ELF) using a model exposure system. Model ELF solutions without (-) and with (+) chelating agents were incubated with 500 μg/mL of CBPs in 1 mL aliquots (37°C) for 0-6h under mixed conditions. At set intervals throughout the exposure, CBPs were removed by centrifugation, and samples rapidly frozen prior to reduced glutathione (GSH), proteins and protein thiols determination. It has been demonstrated that both albumin and chelating agents can protect oxidation of reduced glutathione induced by carbon black particles.

Key words: air pollutants, respiratory tract lining fluid, albumin, reduced glutathione, proteins thiols

Rezumat. În acest studiu am examinat impactul particulelor de cărbune (CBP) tip M 120 asupra glutationului redus şi al proteinelor din fluidul tractului respirator folosind un sistem de expunere model. Soluţiile model de ELF, fără (-) sau cu (+) adaos de agenţi chelatori, au fost incubate cu soluţii conţinând 500 μg particule de cărbune pe ml în volume (37°C) de 1 ml, timp de 0-6 ore, în diferite condiţii. La anumite intervale stabilite în timpul expunerii, particulele de cărbune au fost îndepărtate prin centrifugare iar probele au fost imediat îngheţate, înainte de determinarea glutationului, a proteinelor totale şi a tiolilor proteici. S-a demonstrat că atât albumina cât şi agenţii chelatori pot proteja oxidarea glutationului redus indusă de către particulele de cărbune.

Cuvinte cheie: poluanţi din aer, fluidul tractului respirator, albumină, glutation redus, proteine tiolice

INTRODUCTION

The environment contains a wide variety of pollutants generated by human activities, particularly in heavily industrialized and urbanized settings. Recent health concerns have emerged over pollutants generated from this source - particulate matter (PM) in particular, which, according to World Health Organization (WHO) estimation, is linked with half a milion excess premature deaths each year. The upper exposure limit of PM_{10} (particles of less than 10 microns aerodynamic diameter), established by World Health Organisation is 70 $\mu g/m^3$ when the concentration of sulfur dioxide (SO_2) is smaller than 48 ppb. Background levels of PM_{10} may vary from day to day and with time of day, depending on local pollution sources,

weather conditions and traffic emissions.

A significant part of these pollutants consists of emissions derived from burning of gasoline, diesel fuel, and lubricants in internal combustion engines. Diesel engines have recently gained wider usage because of their efficiency, robustness, and low running costs, and until recently were thought to be more environmentally friendly on account of considerably lower emission levels of carbon monoxide, carbon dioxide and hydrocarbons (1). Diesel engine exhaust contains soot particles with adsorbed many organic and inorganic substances onto them while carbon black is a soot particle analogue virtually free of organic compounds. The carbon black particles are served as a surrogate for the inorganic elemental carbon core particles in Diesel engine exhaust (2). Surface and bulk chemical analysis of PM_{10} indicates that it is a complex mixture of inorganic compounds and organic micropollutants, the composition of which may vary from city to city or between urban and rural environments. Inhalation of ambient air particulate matter is associated with pulmonary injury and inflammation. Epidemiological studies have demonstrated an association between elevated levels of PM_{10} and exacerbation of asthma symptons. Moreover, particulate matter air pollution has been associated with morbidity and mortality from ischemic heart disease and stroke in humans (3). PM_{10} is also associated with a decline in peak expiratory flow rate and increased respiratory symptoms such cough and wheezing in asthmatic children (4).

The purpose of this study was to examine the impact of carbon black particles (CBPs) type M120 on the reduced glutathione and proteins in the respiratory tract epithelial lining fluid (ELF) using a model exposure system.

MATERIALS AND METHODS

Unless stated otherwise, chemicals used in the following protocols were obtained from Sigma (Sigma Chemical Co., St. Louis, MO) or British Drug House (Poole, UK).

Characteristics of particles

The CBPs - type M120 used in this study were donated by Cabot (Billerica, MA). Before the particles were utilized, their morphology and surface chemistry were established. The diameter of single carbon black type M120 was 50 nm and the surface area was 32 m^2/g. All particles tended to aggregate to form bunches or chains in solution (5).

Preparation of CBPs stock solution

The stock CBPs was prepared as follows: 100 mg of dry CBPs were suspended in 10 ml of 0.9% (w/v) saline adjusted to pH 7.4 at room temperature. This solution was continuously mixed during two hours and then 50 µl of mixture were added to desired 1 ml aliquots of sample in multi-well plates giving the final CBPs concentration of 500 µg/ml. To the control samples which did not contain CBPs and were ran in parallel throughout the exposure, 50 µl of 0.9% (w/v) saline at pH 7.4 were added.

Determination of reduced glutathione

Reduced glutathione (GSH) was measured using the enzyme recycling assay (6). Sample concentrations were determined with reference to a standard curve for GSH of 0-6.6 mM. Detection limits was 50 nM. Analyses were performed within 7 days post exposure.

Determination of protein concentration

The method used for the determination of total protein concentrations was based upon a modification of the Lowry method (7) proposed by Smith et al. (8) and adapted for use on a microplate reader. The assay is based upon the observation that when incubated with the cupric ion in an alkaline environment, proteins will reduce Cu (II) to Cu (I) in a concentration dependent manner. In this assay bicinchonnic acid, a highly specific chromogen reagent for Cu (I) was substituted for the Folin-Ciocalteau reagent used in the Lowry assay. Bicinchonnic acid reacts with Cu (I) to form a stable purple complex which has a maximum absorption at 562 nm, the intensity of which is directly proportional to protein concentration over a broad concentration range.

Determination of protein thiols

Protein thiols, the majority of which are present on albumin, were assayed by the method of Ellman (9) according to the protocol of Wayner et al. (10).

Sample solutions

Physiological ELF concentration of 412 μM GSH (11) was prepared as a composite mixture of the above moiety with human albumin at final concentration of 10 mg/mL. Solutions were prepared in 0.9% (w/v), both in the presence and absence of the chelating agents, ethylenediaminetetra-acetic acid (EDTA) and desferoxamine mesylate (DES) (0.1 mM final concentration). Moreover, solutions of pure human albumin at final concentration of 10 mg/mL were prepared in the same manner. Solutions were also adjusted to pH 7.4, to reflect normal human airway secretion pH (12).

Exposure conditions

Exposures were carried out in a 5.6 L perpex chamber. The chamber was maintained throughout at 37±2.8 ^{o}C, and the whole apparatus mounted on an orbital shaker to facilitate mixing of the aqueous phase. Sample solutions were exposed as 1 mL aliquots in multi-well plates (Becton Dickinson UK Ltd., Oxford, UK.) fixed within the exposure chamber. Each well had a diameter of 1.5 cm, giving an exposed surface area of 1.78 cm^2. Prior to commencement of exposure solutions were allowed to equilibrate at 37^{o}C. At this point three samples (time 0 controls) were withdrawn and snap frozen in liquid nitrogen. Particulate were then added to specific cells at defined concentrations to obtain final concentrations of 500 μg/mL. The chamber was then closed, and the orbital shaker was switched on and the experiment started. At set intervals during the exposure: 30, 60, 120, 240, 360 min, the shaker was switched off, the chamber opened and 3 samples removed, again immediately transferred to liquid nitrogen. After

exposure, samples were transferred to -80°C for longer term storage.

Correction for evaporative loss

Due to evaporation, sample volume decreased approximately 15% over 6 h. To correct for this change in concentration, the concentration of Na^+ in control and incubated with CBPs samples was determined using gas-blood analyzer. Then, the increase in Na^+ concentration with time was used as a correction index.

RESULTS AND DISCUSSION

Time related changes in protein concentration during exposure of albumin solutions (10 mg/mL) without and with reduced glutathione (GSH; 400 μmol/L) to 500 μg/mL of ultrafine carbon black particles (CBPs) without chelating agents are shown in Figure 1.

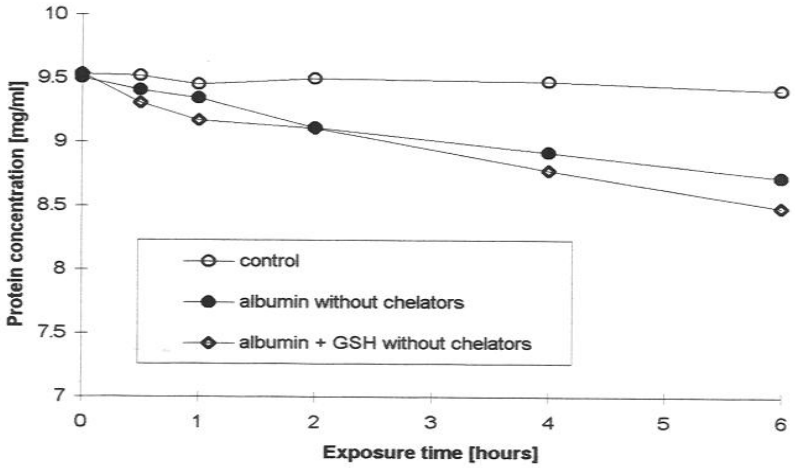

Figure 1. Time related changes in protein concentration during exposure of albumin solutions (10 mg/mL) without and with reduced glutathione (GSH; 400 μmol/L) to 500 μg/mL of ultrafine carbon black particles (CBPs) without chelating agents. The results are the mean of three replications.

The linear decrease in albumin concentration was observed up to six hours of exposure either pure albumin solution or a mixture with reduced glutathione. The highest decrease was found after six hours after exposure. It was about 7-9% of control sample values found at that time point. No differences in protein concentration were found between exposure of pure albumin and mixture of albumin with reduced glutathione to CBPs. It clearly indicates at adsorption of albumin onto the surface of CBPs. When the chelating agents were added to the investigated system, no changes in albumin concentration were noted (Figure 2).

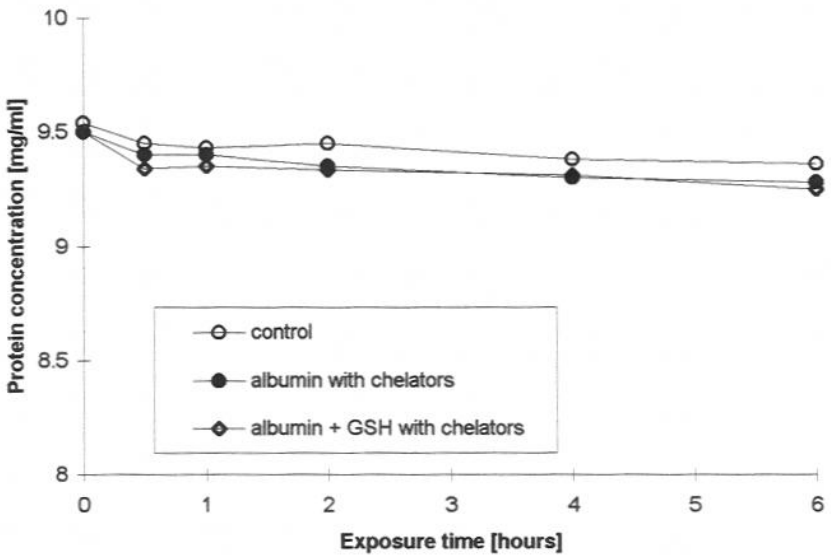

Figure 2. Time related changes in protein concentration during exposure of albumin solutions (10 mg/mL) without and with reduced glutathione (GSH; 400 μmol/L) to 500 μg/mL of ultrafine carbon black particles (CBPs) with 0.1 mM EDTA + DES. The results are the mean of three replications.

This finding may indicate that adsorption of different macromolecules onto the surface of CBPs has a competitive action between albumin and added chelating agents. It has been discussed that size of origin and soure of origin (13), number of particles and total mass (14) and their available reactive surface area (15) can make a significant contribution to health outcome. The available reactive surface area is strongly connected with particle size and is a very important factor, when we consider possible reactions of CBPs with protective lung lining fluids or with many types of epithelial cells lining the respiratory tract (16).

The next question which was addressed in this study was to find out a quality changes of albumin during exposure to CBPs. In order to resolve it, the protein thiols were measured during the exposure period. It was shown that protein thiols were decreased during exposure of albumin solutions (10 mg/mL) without and with chelating agents (0.1 mM EDTA + DES) to 500 μg/mL of ultrafine carbon black particles (CBPs). The higher decrease was found in samples which did not contain added chelating agents (Figure 3).

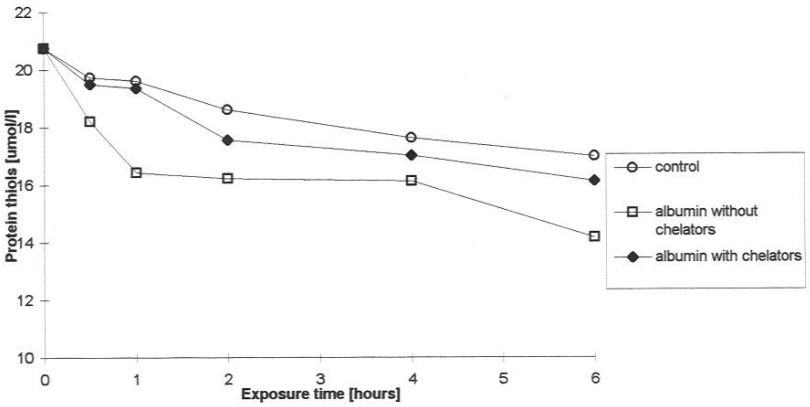

Figure 3. Time related changes in protein thiol concentration (μmol/L) during exposure of albumin solutions (10 mg/mL) without and with chelating agents (0.1 mM EDTA+ DES) to 500 μg/mL of ultrafine carbon black particles (CBPs). The results are the mean of three replications.

These findings indicate that at least three factors can be responsible for the observed decrease as follows: (1) oxidation of sulphydryls by oxygen contained in the samples, (2) oxidation of sulphydryls by the trace metal ions contained in the samples, (3) reaction occuring on the reactive surface of CBPs but depending on free reactive area of particles. The same observations were noted after determination of total thiols during exposure of albumin solutions (10 mg/mL) containing reduced glutathione (GSH; 400 μmol/L) without and with chelating agents (0.1 mM EDTA + DES) to 500 μg/mL of ultrafine carbon black particles (CBPs) (Figure 4).

It can be concluded that besides of previously mentioned three factors responsible for the observed decrease in total thiols, an additional one may be considered. This factor arises from the reaction between oxidized protein thiols and reduced glutathione. It is a well recognized fact that glutathione keeps the protein thiols in the reduced state. However, at the moment, it is not known wheather adsoption properties of albumin onto CBPs surface are better or no in the reduced or oxidized state of albumin.

More clear evidence on the role of albumin and chelating agents in CBPs reactivity was provided by the determination of reduced glutahione concentration during exposure of albumin solutions (10 mg/mL) containing reduced glutathione (GSH;

400 μmol/L) without and with chelating agents (0.1 mM EDTA + DES) to 500 μg/mL of CBPs. It was shown that both albumin and chelating agents can protect reduced glutathione oxidation induced by particles but albumin seems to be more important in this respect (Figure 5).

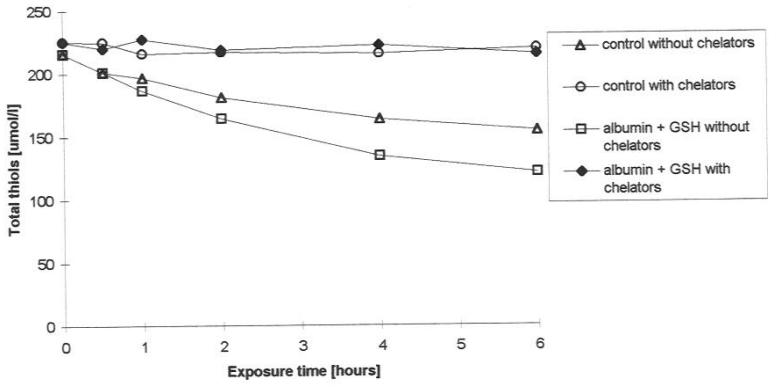

Figure 4. Time related changes in total thiol concentration (μmol/L) during exposure of albumin solutions (10 mg/mL) containing reduced glutathione (GSH; 400 μmol/L) without and with chelating agents (0.1 mM EDTA + DES) to 500 μg/mL of ultrafine carbon black particles (CBPs). The results are the mean of three replications.

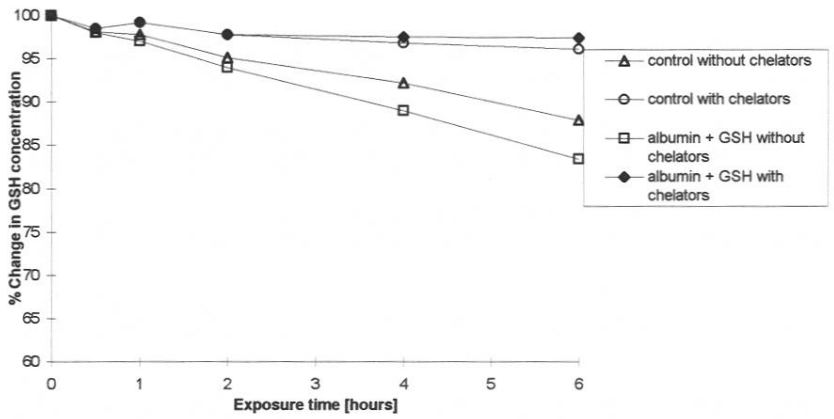

Figure 5. % Changes in GSH concentration during exposure of albumin solutions (10 mg/mL) containing reduced glutathione (GSH; 400 μmol/L) without and with chelating agents (0.1 mM EDTA + DES) to 500 μg/mL of ultrafine carbon black particles (CBPs). The results are the mean of three replications.

This finding is more important in respect to recent evidences which describe complex consumption kinetics for reduced glutathione (GSH), with a rapid initial loss after exposure to 500μg/ml CBPs, followed by the establishment of a new steady state concentration (17).

In the context of defense of the airway and underlying tissues the composition of the respiratory tract lining fluid (RTLF) becomes important. RTLF contains an array of antioxidants such as vitamin C, uric acid, reduced glutathione, alpha-tocopherol, chelating metal ion proteins and antioxidant enzymes (11,18,19). Together, they make up an integrated antioxidant system within the lung, which forms the first line of defense against inhaled atmospheric pollutants such as ozone, nitrogen dioxide and small particulate matter (16). As these pollutants act via free radical mechanisms, an individual's complement of RTLF antioxidants will dictate, in part, their response to air pollutants (20).

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