Blood Cells, Molecules and Diseases 52 (2014) 166–174

Contents lists available at ScienceDirect

Blood Cells, Molecules and Diseases

journal homepage: www.elsevier.com/locate/bcmd

Cigarette smoke induces alterations in the drug-binding properties of human serum albumin

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ARTICLE INFO

Article history:
Submitted 24 July 2013
Available online 3 January 2014

(Communicated by R. I. Handin, M.D.,
3 December 2013)

Keywords:
Cigarette smoke
Human serum albumin
Oxidation
Protein conformation
Naproxen
Salicylic acid

ABSTRACT

Albumin is the most abundant plasma protein and serves as a transport and depot protein for numerous endogenous and exogenous compounds. Earlier we had shown that cigarette smoke induces carbonylation of human serum albumin (HSA) and alters its redox state. Here, the effect of whole-phase cigarette smoke on HSA ligand-binding properties was evaluated by equilibrium dialysis and size-exclusion HPLC or tryptophan fluorescence. The binding of salicylic acid and naproxen to cigarette smoke-oxidized HSA resulted to be impaired, unlike that of curcumin and genistein, chosen as representative ligands. Binding of the hydrophobic fluorescent probe 4,4′-bis(1-anilino-8-naphtalenesulfonic acid) (bis-ANS), intrinsic tryptophan fluorescence, and susceptibility to enzymatic proteolysis revealed slight changes in albumin conformation. These findings suggest that cigarette smoke-induced modifications of HSA may affect the binding, transport and bioavailability of specific ligands in smokers.

Introduction

Human serum albumin (HSA) is the most abundant protein in human plasma and accounts for 50–60% of total plasma proteins [1]. An important amount of albumin is localized extravascularly and represents more than double of the intravascular pool [1]. Albumin contains 17 disulphide bonds and one free cysteine thiol group that of Cys34 [1]. HSA reaches concentrations of more than 0.6 mM (mean ~43 $mg \cdot ml^{-1}$, range 35–52 $mg \cdot ml^{-1}$) in healthy humans. As a consequence, the Cys34 thiol moiety confers a major role in plasma antioxidant capacity to albumin, which accounts for 80% (~500 μmol/l) of the thiols in plasma [2].

In healthy young people, 70–80% of total HSA contains the free sulphydryl group of Cys34 (human mercaptalbumin, HSA-SH). About 25% of albumin forms a reversible mixed disulphide with low molecular weight thiols, such as cysteine, cysteinylglycine, and glutathione [3–5]. A minor fraction (2–5%) also exists to higher oxidation states, i.e., the sulphinic (HSA-SO_2H) or sulphonic acid (HSA-SO_3H) form [6–8]. The oxidized forms of albumin (nonmercaptoalbumins) are picked up in the circulation since they are not present in albumin secreted from the liver cells [9].

Amongst its several physiological and pharmacological functions, albumin can bind a wide variety of endogenous and exogenous compounds [10]. The distribution, free concentration and transport of various ligands significantly depend on their binding to HSA [9]. The binding/transport properties of albumin, in turn, depend on the three dimensional structure of its binding sites, which are distributed over the molecule [9]. It is conceivable that any alteration of the structure and conformation of HSA by some xenobiotic might result in impairment of binding and transport of essential ligands, affecting their overall distribution and efficacy.

The water soluble oxidants of cigarette smoke can readily reach both the systemic circulation [11] and thus they can directly promote vascular oxidative stress in systemic vascular beds [12], leading to various smoke-related degenerative diseases. One cause that had been considered for the pathogenesis of most of the degenerative diseases is oxidative damage of proteins. Increased levels of oxidized proteins have been

Abbreviations: bis-ANS, 4,4′-bis(1-anilino-8-naphtalenesulphonic acid); CSE, whole-phase cigarette smoke extract; DTT, dithiothreitol; HSA, human serum albumin; HSA-SH, human mercaptalbumin; PBS, phosphate buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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http://dx.doi.org/10.1016/j.bcmd.2013.12.001

found in serum of smokers [13–16] and albumin was shown to be the major oxidized (carbonylated) protein in the bronchoalveolar lavage fluid in older smokers [17,18]. Parenchymal lung tissue of current smokers with chronic obstructive pulmonary disease contained lower levels of total albumin, but had proportionally greater levels of highly oxidized (carbonylated) albumin, compared to patients with normal lung function [19]. Recently, we have investigated the susceptibility of HSA to α,β-unsaturated aldehyde-induced carbonylation when exposed to whole-phase cigarette smoke extract [20]. Our results showed depletion of albumin Cys34 free thiol and marked carbonylation. Michael adducts with α,β-unsaturated aldehydes were detected at Cys34, Lys525, and Lys351, whereas His39, Lys541, and Lys545 were found to form a Schiff base with acrolein [20].

Although protein oxidation has been suggested to be of pathophysiological relevance for smokers [13–19], there is no direct proof that oxidation of HSA induced by cigarette smoke leads to changes in its drug-binding properties. Therefore, in this study we investigated whether whole-phase cigarette smoke extract (CSE), a widely used model system to study in vitro effects of cigarette smoke [12,21–23], alters ligand binding properties of HSA.

Materials and methods

Materials

Delipidized crystalline HSA (~99% agarose gel electrophoresis), trypsin from bovine pancreas (EC 232-650-8, BAEE 13,500 units/mg protein), subtilisin® *Carlsberg* (EC 232-752-2, BAEE 11 units/mg protein), salicylic acid, naproxen, curcumin, and genistein were purchased from Sigma-Aldrich (Milan, Italy). 4,4′-Bis(1-anilino-8-naphtalenesulphonic acid) (bis-ANS) was purchased from Sigma-Aldrich (Milan, Italy). All other reagents were of analytical grade. Research-grade cigarettes (3R4F) were purchased from the College of Agriculture c/o Kentucky Tobacco Research & Development Center, University of Kentucky (USA).

Preparation of human mercaptalbumin (HSA-SH)

Delipidized HSA (12 mg/ml, 0.18 mM) was quantitatively converted to HSA-SH, in which the single Csy34 thiol is completely reduced, by treatment with 1.5 mM dithiothreitol (DTT) in 50 mM potassium phosphate buffer (PBS), pH 7.4, for 15 min at room temperature. The excess of DTT was then removed by exhaustive dialysis against 50 mM PBS, pH 7.4.

Preparation of whole-phase cigarette smoke extract (CSE)

Whole-phase CSE from Kentucky 3RF4 reference cigarettes was prepared as previously described [20]. Mainstream smoke from one cigarette (10 puffs) was allowed to dissolve (for 10 s each puff) in 1 ml of 50 mM PBS, pH 7.4. The resultant dark yellow solution was defined as 100% whole-phase CSE and was filtered through a 0.22-mm Millipore filter (Bedford, MA) to remove bacteria and large particles. The pH of the whole-phase CSE was adjusted to 7.4 by the addition of 2 M sodium hydroxide solution. To ensure standardization between experiments and batches of CSE, CSE preparations were made uniform by measurement of absorbance at 340 nm. CSE was freshly prepared immediately before use for each experiment and diluted to an appropriate concentration with 50 mM PBS.

Exposure of HSA-SH to CSE

HSA-SH concentration was determined according to the Bradford protein assay [24]. HSA-SH (4 mg/ml, 60 μM) was treated for 60 min, at 25 °C, with different concentrations of CSE (i.e., 4% and 16%, v/v), with gentle rotary shaking. The removal of CSE was accomplished by exhaustive dialysis against PBS at 4 °C. As the whole-phase CSE reveals an intrinsic fluorescence when measured by using an excitation wavelength of 340 nm and an emission wavelength of 440 nm, at 25 °C, complete CSE removal was checked by measuring its related fluorescence in the dialysis buffer [20].

Evaluation of ligand binding constants by equilibrium dialysis and size-exclusion HPLC

Salicylic acid was dissolved in 20 mM Tris–HCl, pH 7.4, in order to obtain a 10 mM stock solution. Naproxen was dissolved in 20 mM NaOH, diluted 7 mM with 20 mM Tris–HCl, pH 7.4 and the pH adjusted with HCl. Finally, the concentrated naproxen solution was diluted with 20 mM Tris–HCl, pH 7.4, to a stock concentration of 3.5 mM. Equilibrium dialyses were prepared as follows: internal protein solution (1-ml aliquots of 10 μM control or CSE-treated HSA-SH in 20 mM Tris–HCl, pH 7.4) was enclosed in a 45 kDa molecular cut-off dialysis tube, while external buffer (9 ml of 20 mM Tris–HCl, pH 7.4) contained increasing drug concentrations, ranging from 1 to 400 μM. After overnight equilibration with gentle rotary shaking at 4 °C, internal and external solutions were collected and analyzed by gel-filtration HPLC in order to calculate protein and ligand concentrations inside and outside the dialysis membrane, as detailed below. Samples were charged onto a BioSep S4000 Phenomenex 300 × 7.8 mm column and eluted with a 0.5% SDS solution in MilliQ water. Albumin content was estimated by means of absorbance at 215 nm, while drug concentrations were evaluated by fluorescence emission intensity. According to previous papers [25,26], for naproxen and salicylic acid, excitation wavelengths were set at 280 and 295 nm, respectively, while emission intensity was recorded at 310 and 400 nm, respectively, using a Kontron SFM-25 spectrofluorometer.

Evaluation of ligand binding constant by tryptophan fluorescence

Curcumin (1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiens-3,5-dione) and genistein (4′,5,7-trihydroxy isoflavone) were solubilized in 50% ethanol obtaining, respectively, a 3.2 mM and 0.8 mM stock solution. A solution of 25% ethanol doesn't alter albumin structure [27], so samples were prepared maintaining this final ethanol concentration. For both ligands, control and CSE-treated HSA-SH samples contained 0.2 mg/ml (i.e., 3 μM) albumin (final concentration) and an increasing ligand concentration (5–10–20–40–80 μM). After 1-h incubation in the dark, albumin Trp214 fluorescence emission spectra were acquired at 25 °C by using an excitation wavelength of 290 nm and scanning at emission wavelengths from 250 to 450 nm. Fluorescence data, collected with a Kontron SFM-25 spectrofluorometer by using 10 × 10 mm acrylic cuvettes, were used to evaluate curcumin and genistein binding parameters to albumin (K_a = binding constant and n = number of ligand molecules bound).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Control and CSE-treated albumin samples were mixed with 2× SDS sample buffer, without reducing agents and then subjected to SDS–PAGE. In a parallel set of samples, 50 mM DTT was added to the SDS sample buffer. Electrophoresis was carried out using Criterion Tris–HCl 12.5% resolving gels (Bio-Rad Laboratories, Hercules, CA, USA), which were then stained with Coomassie Brilliant Blue.

Fluorescence and UV–vis absorbance spectroscopy

For measurement of intrinsic tryptophan fluorescence, control or CSE-treated HSA-SH was diluted to 0.2 mg/ml (i.e., 3 μM, final concentration) in 50 mM PBS, pH 7.4, kept in the dark at room temperature and tryptophan fluorescence was measured 30 min later. Albumin Trp214 fluorescence emission spectra were determined at 25 °C by using an excitation wavelength of 290 nm and emission intensity was