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Interaction of *p*-benzoquinone with hemoglobin in smoker's blood causes alteration of structure and loss of oxygen binding capacity

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ABSTRACT

Cigarette smoke (CS) is an important source of morbidity and early mortality worldwide. Besides causing various life-threatening diseases, CS is also known to cause hypoxia. Chronic hypoxia would induce early aging and premature death. Continuation of smoking during pregnancy is a known risk for the unborn child. Although carbon monoxide (CO) is considered to be a cause of hypoxia, the effect of other component(s) of CS on hypoxia is not known. Here we show by immunoblots and mass spectra analyses that in smoker's blood *p*-benzoquinone (*p*-BQ) derived from CS forms covalent adducts with cysteine 93 residues in both the β chains of hemoglobin (Hb) producing Hb-*p*-BQ adducts. UV–vis spectra and CD spectra analyses show that upon complexation with *p*-BQ the structure of Hb is altered. Compared to nonsmoker's Hb, the content of α-helix decreased significantly in smoker's Hb ($p = 0.0224$). *p*-BQ also induces aggregation of smoker's Hb as demonstrated by SDS-PAGE, dynamic light scattering and atomic force microscopy. Alteration of Hb structure in smoker's blood is accompanied by reduced oxygen binding capacity. Our results provide the first proof that *p*-BQ is a cause of hypoxia in smokers. We also show that although both *p*-BQ and CO are responsible for causing hypoxia in smokers, exposure to CO further affects the function over and above that produced by Hb-*p*-BQ adduct.

1. Introduction

Cigarette smoking has been identified as the most important source of morbidity and mortality worldwide. The Centers for Disease Control (CDC) estimate that smoking will result in death or disability for half of all people who continue to smoke [1]. Generally the life expectancy of smokers is nearly 14 years less than nonsmokers [2–5]. Apart from causing the various life-threatening diseases such as cancer of the lung and other organs, chronic obstructive pulmonary disease (COPD) and cardiovascular disease (CVD), cigarette smoke (CS) is also known to produce hypoxia [6,7]. In states of oxygen deficiency, the cells, tissues and the body as a whole are deprived of vital energy. Chronic hypoxia may be a cause of early aging, morbidity and premature death. Moreover, CS-related degenerative diseases such as cancer, COPD and CVD are likely to be further complicated by hypoxia resulting in shortening of life-span. Also, smoking during pregnancy causes reduced availability of oxygenated blood to the fetus resulting in intrauterine hypoxia and various risks for the unborn child [8–11]. However, the molecular mechanisms of cigarette smoke (CS)-induced hypoxia are not clear.

Cigarette smoke (CS) is a complex mixture of harmful chemicals, including long-lived semiquinones and carbon monoxide (CO) [12,13]. CO forms carboxyhemoglobin (COHb) in smoker's blood and this may be one of the mechanisms of hypoxia in heavy smokers [13]. However, the formation of COHb is a reversible process. The half-life of COHb of CO-poisoned patients treated with 100% oxygen at atmospheric pressure is about 74 ± 25 min [14]. Earlier we had shown that irrespective of the source, CS contains substantial amounts (100–200 μg/cigarette) of *p*-benzosemiquinone (*p*-BSQ) [15,16]. In the smoker's lung, *p*-BSQ is converted to *p*-benzoquinone (*p*-BQ), which is a strong arylating agent [17,18]. *p*-BQ produced in the smoker's lungs gets into the blood stream and forms covalent adducts with ε-amino groups of lysine residues of human serum albumin (HSA) [18]. This results in alteration of structure and ligand binding capacity of HSA [18]. Being a hydrophobic compound, *p*-BQ readily enters the cell [19]. We conceive that in the smoker's blood,

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p-BQ would enter into the red blood cells (RBC) and form covalent adduct with hemoglobin (Hb), which might result in alteration of its structure. The structure of Hb is so delicately balanced that small structural change such as modifications of internally located amino acid residues may render it nonfunctional for carrying oxygen [20]. We further envisage that the associated structural changes, if any, might alter the oxygen binding capacity, resulting in reduced oxygen delivery to the tissues leading to hypoxia. Since smokers are also exposed to CO [13], we were interested to see the interaction of CO with *p*-BQ on oxy-Hb.

2. Materials and methods

2.1. Materials

Human Hb was purchased from Sigma (H7379) and used without further purification. *p*-Benzoquinone (*p*-BQ) was obtained from Himedia (RM-489) and freshly crystallized from *n*-hexane before use. All other reagents used were of analytical grade. Affinity purified polyclonal antibody to *p*-BQ raised in rabbit after immunization with *p*-BQ-bovine serum conjugate was supplied by Abexome Biosciences, Bangalore, India. LC/MS- grade solvents, acetonitrile and formic acid were purchased from Fluka. RapiGest™. Sodium iodide (NaI), polyethylene glycol (PEG) mix and Glu-fibrino peptide B (GFP) were obtained from Waters (Milford, MA, USA). Trypsin was purchased from Sigma–Aldrich (St. Louis, MO, USA). Ammonium bicarbonate and ammonium acetate were purchased from Merck (India). All other reagents used were of analytical grade.

2.2. Ethics statement

The collection of human blood and subsequent experiments were approved by the Institutional Bioethics Committee for animal and human research studies, University of Calcutta, following the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Written consents were obtained prior to collection of blood.

2.3. Preparation of *Hb*-p-*BQ adduct*

Hemoglobin was incubated with *p*-BQ in definite molar ratios (1:1, 1:2, 1:10, 1:20, 1:50) in 20 mM potassium phosphate buffer (pH 7.4) for two hours at 37 °C in the dark. The reaction mixture was dialyzed overnight at 4 °C to remove excess/free *p*-BQ and hydroquinone formed as a reaction product. The stock solution was diluted and used for subsequent experiments.

2.4. Isolation of hemoglobin from of human blood

Red blood cell (RBC) was isolated from whole blood using Ficoll-Paque™ PLUS reagent (Amersham Biosciences) following standard protocol and the pellet was washed with phosphate buffered saline (PBS) thrice. RBC was lysed by adding equal volume milliQ water to the pellet. The resulting solution was centrifuged at 20,000 × *g* for 15 min at 4 °C and the supernatant purified through PD-10 Desalting Column followed by Centricon centrifugation using 30 k Da membrane and the purified Hb was used for subsequent experiments.

For mass spectra analysis, Hb was isolated following the method described elsewhere [21]. Briefly, venus blood from smokers, collected in EDTA vial, was centrifuged at 1248 × *g* for 10 min at 25 °C. The obtained packed cells was washed with 0.9% NaCl thrice and then lysed with eight volumes of ice cold distilled water. The hemolysate was centrifuged at 12,880 × *g* for 10 min at 4 °C to remove the erythrocyte membranes. The Hb solution was then lyophilized, stored at −20 °C and used for mass spectroscopic analysis when needed.

2.5. Spectrophotometry of *Hb*-p-*BQ adduct*

The stock Hb solution prepared as described above was diluted to desired concentration and the absorbance was measured from 200 to 700 nm in a Shimadzu UV-2540 spectrophotometer. For time-dependent measurement of Hb-*p*-BQ interaction, Hb was incubated with *p*-BQ at a molar ratio of 1:50.

2.6. Mass spectrometric analysis of *Hb*-p-*BQ adduct A. Intact protein analysis*

Samples were prepared and dialyzed overnight against 10 mM ammonium acetate, pH 7.4. Immediately before the mass spectroscopic analysis, the lysate was diluted to a concentration of 25 μM in 10 mM ammonium acetate, pH 7.4. Using gold coated borosilicate capillaries, the sample was infused into nano-ESI source of Synapt HDMS mass spectrometer (Waters, Manchester, UK). Data were acquired in positive ion mode with a capillary voltage of 1.9 kV in the *m*/*z* range 650–5000. TOF analyzer was calibrated using cesium iodide (2 mg/ml) in 50% aqueous 2-propanol. The instrument acquisition parameters were adjusted to obtain optimal signals. The sampling and extraction cone voltages were set to 100 V and 2 V respectively. Source temperature was maintained at 37 °C and the gas flow was set at 1.5 ml/min. The backing pressure was increased to 5.9 m bar for better transmission of large protein assemblies aided by collisional cooling. Data were acquired and processed with MassLynx v4.1 software (Waters, UK).

2.7. Globin chain analysis

2 μg of hemolysate protein was injected through a C18 RP analytical column (ZORBAX Eclipse, 150 mm × 4.6 mm, 3.5 μm) at room temperature. Globin chains were eluted using a linear gradient of 2% increase in acetonitrile per minute containing 0.1% acetic acid at a flow rate of 0.2 ml/min and the mass analysis was performed on Synapt HDMS with electrospray ionization (ESI) source (Waters). The data were acquired in positive ion 'V' mode over the range of 650–1500 *m*/*z*, with a capillary voltage of 3 kV using a source temperature of 120 °C and desolvation gas temperature of 350 °C. The mass calibration was done using NaI. The mass spectrum was smoothed, baseline subtracted, and subsequently deconvoluted using MaxEnt1 software. Since the charge state distributions of β-globin chain and its adducts were observed to be identical in mass spectra, the respective signal intensities of different molecular ions were correlated with their relative abundance. The most intense signals of the observed protonated populations, namely, β, glycated-β, glutathionylated-β and pBQ adduct of the β-globin chain were found to be distributed across the charge states from 11 to 19. Thus the quantification of each population of β-globin adducts was calculated as follows:

$$p\text{BQ} - \beta \text{ adduct of hemoglobin\%} = \frac{p\text{BQ adduct of } \beta \times 100}{(\beta + \text{Hb}\beta - \text{gly} + \text{Hb}\beta - \text{GS} + p\text{BQ adduct of } \beta)} \quad (1)$$

$$\text{Hb}\beta - \text{gly\%} = \frac{\text{Hb}\beta - \text{gly} \times 100}{(\beta + \text{Hb}\beta - \text{gly} + \text{Hb}\beta - \text{GS} + p\text{BQ adduct of } \beta)} \quad (2)$$

$$\text{Hb}\beta - \text{GS\%} = \frac{\text{Hb}\beta - \text{GS} \times 100}{(\beta + \text{Hb}\beta - \text{gly} + \text{Hb}\beta - \text{GS} + p\text{BQ adduct of } \beta)} \quad (3)$$