Publications


Cigarette smoke induces p-benzoquinone–albumin adduct in blood serum: Implications on structure and ligand binding properties

Arunava Ghosh¹, Aparajita Choudhury⁴, Archita Das⁴, Nabendu S. Chatterjee⁵, Tanusree Das⁵, Rukhsana Chowdhury⁶, Koustitha Panda⁴, Rajat Banerjee⁴**, Indu B. Chatterjee⁴* *

¹ Department of Biotechnology and Dr. B. C. Gaha Centre for Genetic Engineering & Biotechnology, Calcutta University College of Science, 35. Ballygunge Circular Road, Kolkata 700019, India
² National Institute of Cholera and Enteric Diseases, P-33, C.I.T. Road, Scheme-VM, Beliaghata, Kolkata 700010, India
³ Indian Institute of Chemical Biology, 4, Raja S. C. Mullick Road, Kolkata 700032, India

ABSTRACT

Earlier we had reported that irrespective of the source cigarette smoke (CS) contains substantial amounts of p-benzenesemiquinone, which is readily converted to p-benzoquinone (p-BQ) by disproportionation and oxidation by transition metal containing proteins. Here we show that after CS-exposure, p-BQ-protein adducts are formed in the lungs as well as serum albumin of guinea pigs. We also show that serum of human smokers contains p-BQ-albumin adduct. It is known that human serum albumin (HSA) plays a very important role in binding and transport of a variety of ligands, including fatty acids and drugs. We show in vitro that p-BQ forms covalent adducts with free amino groups of all twenty amino acids as well as e-amino groups of lysine residues of HSA in a concentration dependent manner. When HSA is incubated with p-BQ in the molar ratio of 1:1, the number of p-BQ incorporated is 1. At the molar ratio of 1:60, the number of p-BQ incorporated is 40. The formation of HSA–p-BQ adduct has been demonstrated by absorption spectroscopy, MALDI-MS and MALDI-TOF-TOF-MS analyses. Upon complexation with p-BQ, the secondary structure and conformation of HSA are altered, as evidenced by steady state and time-resolved fluorescence, circular dichroism, 8-anilino-1-naphthalenesulfonic acid binding and differential scanning calorimetry. Alteration of the structure and conformation of HSA results in impairment of its ligand binding properties with respect to myristic acid, quercetin and paracatetamol. This might be one of the reasons why transport and distribution of lipids and drugs are impaired in smokers.

© 2011 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Numerous scientific and epidemiological evidences have unequivocally established that cigarette smoking is a risk factor for various degenerative diseases like cancer of the lungs and other organs (Bartecchi et al., 1994; Calle et al., 1994; Giovino, 2002; Kuper et al., 2002; Boffetta, 2008; Sequist, 2008), emphysema (Lopez and Murray, 1998; Tudor et al., 2003; Banerjee et al., 2008), and cardiovascular disease (Services, 1989). Even in smokers without any apparent clinical symptoms, the overall life expectancy is reduced, ranging up to 10 years fewer than non-smokers (Doll et al., 2004). One reason that had been considered for the pathogenesis of most of the degenerative diseases is oxidative damage of proteins, lipids and DNA (Cross et al., 1993; Asami et al., 1997; Santanam et al., 1997). The oxidants present mainly in the tar phase (Panda et al., 2001) had been identified to be water soluble semiquinone radicals, particularly, o- and p-benzenesemiquinones (Pryor et al., 1983; Pryor et al., 1998). The water soluble oxidants of CS can readily reach both the systemic circulation (Csiszar et al., 2008) and interstitial cells (Ishii et al., 2001) leading to various degenerative diseases. Subsequently, we isolated the stable oxidants from aqueous extract of CS (AECS) and characterized it as p-benzenesemiquinone (p-BSQ) (Banerjee et al., 2008). We have observed that the mainstream smoke from all commercial cigarettes examined as well as Kentucky research cigarettes contains substantial amounts (100–200 µg/cigarette) of p-BSQ (Chatterjee, 2005; Dey et al., 2010). Under physiological conditions, p-BSQ is readily converted to p-BQ by disproportionation (Sullivan and Reynolds, 1996) and oxidation by transition metal containing proteins (Banerjee et al., 2008). p-BQ is a redox cycling agent that produces reactive oxygen species (ROS) leading...
to oxidation of proteins and DNA (Bolton et al., 2000). Although CS does not contain p-BQ, it is conceivably produced in the smoker’s lungs through oxidation of p-BQ by Cu, Zn-SOD and cytochrome c (Banerjee et al., 2008). Recently, we have reported that AECS-induced formation of ROS and oxidative damage in human lung cells (AS49) is not only completely prevented by antibody to p-BQ, but also mimicked by p-BQ in amounts present in AECS (Dey et al., 2011). This indicates that the causative factor responsible for AECS-induced oxidative damage is p-BQ.

However, besides being a redox cycling agent generating ROS, p-BQ functions as a strong arylation agent producing Michael adducts with proteins (Bolton et al., 2000). Earlier we had shown that p-BQ forms covalent adducts with BSA (Banerjee et al., 2008). We had also shown that p-BQ forms protein adducts in the bone marrow of CS-exposed guinea pigs (Das et al., 2011). This indicates that p-BQ formed in the lungs, gets into the blood stream, and then to different tissues of the body. Thus it would thus be interesting to investigate whether p-BQ interacts with serum albumin in smoker’s blood.

Human serum albumin (HSA) is the principal extracellular protein of blood plasma accounting for about 60% of the total protein (He and Carter, 1992; Dockal et al., 1999). HSA plays a very important role in binding and transport of an unusually broad spectrum of exogenous and endogenous ligands, including fatty acids and a variety of drugs (Bhattacharyya et al., 2000; Ghuman et al., 2005; Varshney et al., 2010). The distribution, free concentration and transport of various ligands significantly depend upon their binding to HSA (Peters, 1996). 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. As mentioned earlier, one such xenobiotic is p-BQ, which is derived mostly from exposure to CS (Banerjee et al., 2008; Das et al., 2011; Dey et al., 2011).

Since p-BQ forms covalent adducts with BSA (Banerjee et al., 2008), it would conceivably form adduct also with HSA. It is possible that in smokers, p-BQ forms covalent adduct with HSA resulting in alteration of structure and impairment of ligand binding, including lipid, which is the primary physiological ligand of the protein (Bhattacharyya et al., 2000). p-BQ-mediated alteration in serum lipid transport may be one of the underpinning mechanisms for accumulation of lipids in serum of smokers. It is reported that smokers have significantly higher serum cholesterol, triglyceride, and low-density lipoprotein levels (Craig et al., 1989).

HSA has long been the centre of attention of also the pharmaceutical industries. The efficacy of a drug depends on the binding with HSA, transport, distribution and the pharmacokinetic properties, including lipids and drugs. Here we show that in vitro that p-BQ forms covalent adducts with e-amino groups of Lys residues of HSA in a concentration dependent manner leading to alteration of HSA’s secondary structure and conformation resulting in impairment of binding with myristic acid. HSA is also known to play an important role in binding and transport of quercitin, a naturally occurring flavonoid having antioxidant and cardioprotective activity as well as paracetamol, a commonly used analgesic and antipyretic drug (Manach et al., 1999; Kaldas et al., 2005; Daneshgar et al., 2009). We further show that bindings of quercitin and paracetamol are altered upon complexation with p-BQ.

2. Materials and methods

2.1. Material

HSA was purchased from Sigma (A-1887 fatty acid free) and used without further purification. p-Demethoxynapthoquinone (p-DNQ) was obtained from Himedia (RM-489) and freshly recrystallized from n-hexane before use. All other reagents used were of AR grade. All the spectrofluorimetric analyses were carried out in a Hitachi F-4000 spectrophotometer using a 10 cm × 10 mm quartz light path quartz cell. Absorbance spectra were taken in a Shimadzu UV-2540 spectrophotometer.

2.2. Ethics statement

All methods were approved by the Institutional Animal Ethics Committee, Permit No. 797/PC/SEA, University of Calcutta. All efforts were made to minimize suffering of the animals. The collection of blood and subsequent experiments with human serum were approved by the Institutional Bioethics Committee for animal and human research studies, University of Calcutta, permission no. 1096, following the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

2.3. Collection of blood from human volunteers (smokers and non-smokers)

Written consents were obtained from all the volunteers prior to collection of blood. All donors used in the study were male and in the age group of 25–35 years. Smokers used in this study had a smoking habit for 5 years. Nonsmokers used as control had no prior history of smoking for the last 10 years. Venous blood was taken and serum was isolated.

2.4. Exposure of guinea pigs to cigarette smoke

The procedure was essentially same as that described before (Banerjee et al., 2008). Briefly, male short hair guinea pigs weighing 350–450 g were fed ad libitum a diet for 7 days to minimize the vitamin C level of the tissues. This is because vitamin C is a potential inhibitor of p-BQ-induced tissue damage (Panda et al., 2000; Banerjee et al., 2008). After 7 days of vitamin C deprivation, the animals were exposed to smoke from 5 Kentucky research cigarettes (30 mg/2 puff) for 30 min. The total duration of exposure to smoke from one puff was 1 min 30 s, allowing the animal 1 min rest in smoke-free atmosphere to breathe air between each puff. The gap between one cigarette and the next was 1 h. The procedure was essentially same as that described before (Banerjee et al., 2008). The smoke chamber used was a vacuum desiccator (2.5 l) with an open tube at the top and a side tube fitted with a stopcock. The cigarette placed at the top was lit and CS was introduced into the chamber containing the guinea pig by applying a mild suction through the side tube for 5 s. Thereafter, the vacuum was turned off and the guinea pig was further exposed to the accumulated smoke for another 45 s. Pair-fed sham controls were subjected to air exposure instead of CS under similar conditions. At the end of the experimental period, the guinea pigs were euthanized using i.p. injection of ketamine hydrochloride (100 mg/kg guinea pigs) and blood and lung tissue were collected for further experiments.

2.5. Incubation of HSA with aqueous extract of CS (AECS) and p-BQ

Aqueous extract of CS (AECS) was prepared as described before (Panda et al., 1999). Briefly, smoke from one Kentucky research cigarette 3R4F was extracted with 1 ml of 50 mM potassium phosphate buffer, pH 7.4. Filtered through 0.22 μm Millipore filter and the pH adjusted to 7.4. The method of preparation of AECS was so devised as to simulate the manner in which the respiratory tract lining fluid is exposed to CS during the process of smoking by humans. The AECS solution thus obtained was used immediately. For experiments with AECS or p-BQ-treated HSA, HSA (1 mg) was incubated with 50 μl of AECS or 5 μg p-BQ in a final volume of 200 μl 20 mM potassium phosphate buffer (pH 7.4).

2.6. Immunoblot analysis

Reaction mixtures containing 15 μg protein equivalent of HSA and lung tissue were resolved on 10% SDS-PAGE and transferred to PVDF membrane (Santa Cruz Biotechnology, Inc., sc-7273) and blocked with 5% non-fat dry milk (Himedia KM-1254). Primary antibody used was a double affinity purified polyclonal antibody raised in rabbit against a conjugate of bovine serum albumin and p-BQ (supplied by Abergene Biosciences Pvt. Ltd., India). Blots were then incubated with goat anti rabbit IgG-HRP secondary antibody (GE Healthcare, 105499). The membranes were finally detected by chemiluminescence (LumiGLO Reagent and Peroxide, Cell Signaling Technology). The HSA western blots showed a 45 kD band for HSA and a 48 kD band for p-BQ-HSA. Western blot analysis of lung tissue showed a 45 kD band for HSA and a 48 kD band for p-BQ-HSA.

2.7. Identification of the mass of intact proteins by MALDI-MS

HSA was incubated with p-BQ at different mol ratios (HSA:p-BQ = 1:1 to 1:50) in 20 mM ammonium bicarbonate buffer, pH 7.4, for 2 h at 37 °C and then dialyzed in the same buffer overnight. The dialyzed material was lyophilized. The lyophilized samples were desorbed in sample driers (100% acetonitrile containing 0.1% TFA)
to obtain a protein concentration of 1 mg/ml and 10 μl of this protein solution was
desalted by reverse-phase ZipTip C18 pipette tips (Millipore, USA) and applied to the
MALDI sample plate and mixed with equal volume of α-cyano-4-hydroxycinnamic acid
(CHCA) matrix directly on the target plate. Samples were allowed to dry for several
minutes before MALDI-TOF MS measurements were performed. Spectra of intact protein
molecules were obtained on an MALDI-TOF/TOF mass spectrometer (Model: 4800 MALDI-TOF-TOF, Applied Biosystems, USA). Mass spectra were recorded in the m/z range between 20 and 20,000Da.

2.8. MALDI-TOF-TOF-MS analysis of HSA-p-BQ adduct in smokers’ serum

Comassie (R-250/BioSafe) stained protein bands of interest were cut manually from SDS-PAGE gel and placed into microcentrifuge tubes. Proteins were digested using In-gel trypic digestion kit (PIERCE) and processed according to the manu-
facturer’s instructions. Briefly, the digested peptides were dried in vacuum and subjected to ZipTipC18 purification for MS analysis. This was followed by iden-
tification of proteins using a MALDI-TOF-MS mass spectrometer (Model: 4800 MALDI-TOF-TOF, Applied Biosystems, USA) equipped with a special analysis soft-
ware GPS. All searches were performed against suitable databases.

2.9. Spectrophotometry of p-BQ conjugates with amino acids and N-acetyl amino acids

Each of the common 20 amino acids and N-acetyl amino acids (500 nmol) was incubated with or without 100 nmol of p-BQ in a final reaction volume of 200 μl for two h at 37 °C in 20 mM potassium phosphate buffer, pH 7.4. The N-acetyl amino acids used were N-acetyl Ala, N-acetyl Cys, N-acetyl Trp, N-acetyl His, N-acetyl His and N-acetyl Lys and N-acetyl Lys, before incubation, the pH of the amino acid solution of Lys, Arg, His and Gua was adjusted to 7.4. The reaction mixture obtained after incubation of the respective amino acid with p-BQ as well as the control was diluted to 1 ml by adding 900 μl of 20 mM potassium phosphate buffer, pH 7.4, and spectra were taken in the range of 200–700 nm.

2.10. Estimation of free amino groups of FISA and HSA-p-BQ adducts by
fluorescamine fluorescence

After incubation of HSA with p-BQ in the molar ratio of 1:1 to 1:60 as described above under MALDI-MS, the reaction mixture was added to 1.35 ml of sodium borate buffer (200 mM, pH 8.5) and mixed with 180 μl of fluorescamine (1 mM in acetone). After incubation at room temperature for 15 min in the dark, fluorescence was measured. Taking the fluorescamine fluorescence of unreacted HSA at 474 nm as 100%, the decrease in fluorescence was measured (Colombo et al., 2010).

2.11. In-solution digestion and identification of peptide-p-BQ complex by
MALDI-TOF-TOF-MS

Ten microgram of HSA p-BQ adduct in the molar ratio of 1:1 was dissolved in 15 μl of 50 mM ammonium bicarbonate (ambic) buffer and in-solution tryptic digests (200 μg trypsin, at 37 °C for overnight) were performed after the reduc-
tion/alkylation of the samples using dithiothreitol (DIT: 100/200 mM at 95 °C for 5 min) and iodoacetamide (IAA: 200 mM at ambient temperature for 20 min in dark).
The digested peptide fractions were vacuum-dried and dissolved in 10 μl of sample buffer (60% acetonitrile containing 0.1% TFA) and desalted by reverse-phase ZipTip C18 pipette tips (Millipore, USA) prior to applying on to the MALDI sample plate. A small fraction (0.5 μl) of the sample was spotted on the well and equal volume of α-cyano-4-hydroxycinnamic acid (CHCA) matrix was overlaid with a hydrophobic mask on each spots and allowed to crystallize at room temperature. MALDI-TOF-MS measurements in the positive reflector mode were performed with an ABI 4800 Proteomics Analyzer (Applied Biosystems, USA). The laser wavelength was 355 nm, and the laser repetition rate was 200 Hz. The mass spectra were externally calibrated with the autodigest peaks of trypsin (Mn, 905.505, 1020.504, 1153.574, 2163.057 and 2273.160 Da). The MS/MS data were acquired and processed using the GPS software and MASCOT or SWISS-PROT were used to search the databases to identify the peptides. The following parameters were used in the searches: trypsin digest (one missed cleavage allowed), mass accuracy 0.2 Da, and a mass tolerance of 50 ppm was used for the peptide search; taxonomy: homo sapien; acetylation of the N-terminus, alkylation of cysteine by carbamidomethylation and oxidation of methionine were considered as possible modifications.

MALDI-TOF-MS measurements in the positive reflector mode were performed with an ABI 4800 Proteomics Analyzer (Applied Biosystems, USA).

2.12. Fluorescence and absorption spectra of the HSA-p-BQ adduct

HSA (131 μmol) was incubated with or without different concentrations of p-
BQ ranging from molar ratio of 1:1 to 1:60 in 20 mM potassium phosphate buffer (pH 7.4) at 37 °C for 2h. After incubation the reaction mixture was subjected to overflow against buffer at 4 °C. To each sample started from hydroxyquinone (HQ), a metabolite of p-BQ produced after interaction with HSA. The dialyzed reaction mixture was used for further studies, for time dependent absorbance spectrum, analysis was incubated with p-BQ (1:60) and small aliquots were drawn from the reaction mixture at different interval of time to prepare a 2 μM solution and absorbance spectrum was acquired.

2.13. Fluorescence and absorption spectra of peptide-p-BQ complex

Each custom-made decapeptide (108 nmol) was incubated with 1.5 μmol of p-
BQ in the molar ratio of 1:5 in 20 mM potassium phosphate buffer, pH 7.4, at 37 °C in a final volume of 200 μl for 2h. Controls were incubated separately using only
peptide. Then the reaction mixture was extracted successively twice with n-butanol (200 μl each time) to remove any unreacted p-BQ or hydroquinone (produced from p-BQ) present in the reaction mixture. The upper layer of butanol was discarded and the aqueous layer obtained after second butanol extraction was used for both spectrophotometry and fluorescence study.

2.14. Fluorescence studies

Unless mentioned otherwise, all the fluorescence spectra were excited at a wavelength of 295 nm for selective excitation of tryptophan residues. The emission spectra were recorded from 310 to 450 nm. The path length of the cuvette was 0.5 mm to minimize the inner filter effect. For the same reason, the protein concentration used was 0.5 μM and the optical densities of the samples were kept below 0.1.

2.15. Fluorescence lifetime

Fluorescence lifetimes were determined from the total emission intensity decay of 10 μM reaction mixture, using a nanosecond time-domain
fluorimeter and operated in the time-correlated single-photon-counting mode. Excitation was provided by a pulsed, high-pressure (1.5 atm) N2 lamp operating at 25 kHz, the FWHM of the excitation pulse profile being 1.3 ns. The N2 emission line at 297 nm was used to excite Trp, while the emission was monitored at 340 nm. Slits of bandwidth 16 nm were used in both excitation and emission channels. Mean fluorescence lifetimes were calculated using the relation \( \tau = \sum n_i \tau_i^2 / \sum n_i \tau_i \), where \( n_i \) represents the fractional contribution to the time-resolved decay of the component having lifetime of \( \tau_i \), the decay curve fitted to three exponential decay with least-square value.

2.16. CD analysis

Reaction mixtures prepared for the measurements of fluorescence and absorption spectra of HSA-p-BQ adducts, as described above, was diluted to 5 μM for far
UV CD analysis and 10 μM for near UV CD analysis. CD spectra were recorded on a Jasco 720 spectropolarimeter by scanning reaction mixtures from 200 to 250 nm using 1 mm path length cuvette and 250 to 320 nm for near UV region using 10 mm cuvette path length. Mean residual ellipticity (MRE) was calculated using the formula, MRE = [100 \times \text{Observed}] / \{10 \times n \times I \} in deg cm2 dmol–1, where \( n \) is the observed ellipticity in millideg and \( I \) is the molar concentration in milligram, \( n \) is the number of amino acids and \( I \) is the path length in cm. The α-helix percentage was calculated using MRE value at 222 nm according to the formula, ([MRE] – 2340)/30300 \times 100 (Chen et al., 1972).

2.17. ANS binding

To 1 μM reaction mixture containing HSA and HSA-p-BQ complex, ANS was added from a stock of 50 mM solution to achieve a final concentration of 10 μM. ANS binding was studied using 420 nm as the excitation wavelength and 482 nm as emission wavelength.

2.18. Differential scanning calorimetry (DSC) analysis

DSC measurements were performed using a nanocalorimeter, (N-DSC II, Calorimetry Sciences Corp, Utah U.S.A.). Pure, dialyzed samples of HSA-p-BQ adducts or native HSA (3 mg/ml; 2210 μM) in phosphate buffer (pH 7.4) were heated from 25 to 100 °C at the rate of 1 °C/min increment using the same buffer as blank. DSC data were corrected for instrument baselines (determined by running the dia-
lysis buffer in both reference and sample cells just prior to placing proteins in the sample cell) and normalized for scan rate and protein concentration. Data analyses were performed with Cpcalc software (Calorimetry Sciences Corp, Utah U.S.A.).

2.19. Ligand binding

2.19.1. Myristic acid

The stock solution of MA (1 mM) was prepared in 20 mM potassium phosphate buffer, pH 7.4, at heating at 50 °C. Followed by cooling to room temperature. Myristic acid was added from the stock solution in 3 µl aliquots up to a final concentration of 51–1 µM native HSA or HSA-p-BQ adduct. The excitation wavelength was 295 nm and the emission recorded at 340 nm.
2.19.2. Quercetin
Since quercetin has substantial optical density at 295 and 340 nm that may cause inner filter effect during titration by quenching method, we employed fluorescence anisotropy method which is suitable for measuring protein-ligand dissociation constant and much less sensitive to inner filter effect than quenching. A 10 mM concentrated stock solution of quercetin was prepared in ethanol. From this stock solution, further diluted stock solution of quercetin was prepared in buffer. A final volume of 500 \mu L of 5 \mu M quercetin was incubated with increasing concentrations of protein up to 50 \mu M. Amount of ethanol in the final incubation system was 1.5%.

2.19.3. Paracetamol

A 50 mM stock solution of paracetamol was prepared by dissolving paracetamol in buffer. The concentration was determined using 10.236 M absorptivity of paracetamol at 243 nm (Abdelalat et al., 2007). Small aliquots were added from diluted paracetamol stock to a 0.5 \mu M free HSA or HSA-p-BQ complex up to a concentration of 1.9 mM. The Tryptophan fluorescence quenching was measured using an excitation wavelength of 295 nm and measuring emission at 340 nm.

2.20. Determination of dissociation constant

2.20.1. Anisotropy measurements

Steady-state anisotropy was recorded with a Hitachi model F-7000 spectrophotometer equipped with a polarization accessory. Excitation of quercetin was done at 400 nm. The fluorescence anisotropy (A) values were obtained using the expression:

\[ A = \frac{(I_h - I_v) - (I_h - I_v) + C}{(I_h - I_v) + C} \]

where \( I_h \) and \( I_v \) are the vertically and horizontally polarized components of the fluorescence emission by vertically polarized light at 400 nm and \( C \) is the sensitivity factor of the detection system. The excitation and emission slits were 5 and 10 nm, respectively. The quercetin concentration was 5 \mu M and HSA or HSA-p-BQ concentrations were varied between 1 (Baretechi et al., 1994) and 50 \mu M. The data were fitted according to the following equation (Heyduk and Lee, 1990):

\[ A = A_0 + (A_0 - A_\infty) (1 + (K_d [L]) \times (1 + P L)^2) \]

where \( A \) is the measured value of anisotropy, \( A_0 \) and \( A_\infty \) are specific values of anisotropy associated with free quercetin and HSA–quercetin or HSA–p-BQ–quercetin complex, respectively. The \( K_d \) value was obtained by fitting the experimental data to the equation by using least-squares method using KYPLOT (Koichi Yoshioka, 1997–2000, version 2.0, beta 13) with \( A_0 \), \( A_\infty \) and \( K_d \) as floating parameters.

2.20.2. Fluorescence quenching method

The values of the dissociation constant (\( K_d \)) for the HSA-ligand complex (PL) was determined by assuming a simple biomolecular binding equilibrium between the HSA (P) and ligand, L, using the nonlinear least-squares method according to the following equation (Takita et al., 1996).

\[ (2)(S/F) = \frac{S + K_d L}{S + K_d L_0} \]

where \( S \) is the total concentration of the ligand, \( L_0 \) is the initial concentration of ligand, \( F \) is the fluorescence intensity change observed at 340 nm when a certain amount of the ligand is added. This is expressed as a percentage of the fluorescence intensity of the HSA, namely \( \%S/F = 100 \times (F_0 - F_1)/F_0 \), where \( F_0 \) and \( F_1 \) are the fluorescence intensities at 340 nm of the HSA-ligand complex and the HSA, respectively. The data were fitted using KYPLOT (Koichi Yoshioka, 1997–2000, version 2.0, beta 13) with \( S/F_{max} \) and \( K_d \) as floating parameters.

3. Results

3.1. Identification of HSA-p-BQ adduct in smokers’ serum

As reported earlier, p-BQ is derived from p-BSQ of CS by disproportionation (Sullivan and Reynolds, 1996) and oxidation by transition metal catalyzed proteins, including Cu, Zn-SOD and cytochrome c (Banejee et al., 2008). Conceivably, p-BQ is expected to be produced from p-BSQ in smokers’ lung, to enter into the blood stream and form adduct with HSA. This is evidenced by immunoblot analysis showing that lungs as well as serum of guinea pigs exposed to CS contain p-BQ–protein adducts (Fig. 1A and B) and we also show that serum from human smokers’ contains p-BQ adduct with protein corresponding to the molecular weight of HSA (Fig. 2). The presence of HSA-p-BQ adduct in smokers’ serum has been confirmed by mass spectrometry. MALDI-TOF-TOF MS data obtained from two smokers (smokers 5 and 6 of Fig. 2) show that altogether eight locations of HSA have formed adduct with p-BQ (Table 1). In separate experiments we show that serum treated with aqueous extract of CS (AECS) or p-BQ using anti p-BQ antibody. Fifteen microgram protein was loaded in each lane. Loadin controls: \( \alpha \)-tubulin in (A) and ponceau S staining of the membrane as the loading control. Details of the procedure are given under Section 2.

3.2. MALDI-MS analysis of HSA-p-BQ adducts

To confirm that p-BQ forms adducts with HSA, we carried out MALDI-MS of HSA incubated in vitro with different concentrations of p-BQ. Upon complex formation with p-BQ, the molecular weight of HSA is expected to increase according to the number of p-BQ adducted. For each p-BQ adduct, an increase in molecular weight (MW) of 108 (m/z) Da should be obtained. From the MALDI mass spectrum of protein and p-BQ complexed protein, the increase in MW is clearly evidenced in accordance with the concentration of p-BQ (Table 2). The number of p-BQ complexed with HSA increased with increased molar ratio of HSA-p-BQ (Table 2). For a 1:1 molar ratio of HSA-p-BQ, the number of p-BQ adducted was 1; for 1:5 ratio, the number was 7; for 1:30 was 24 and for 1:60 it was
Table 1
MALDI-TOF-TOF-MS analysis of HSA–p-BQ adduct in smokers' serum.

<table>
<thead>
<tr>
<th>Amino acid residues</th>
<th>MW of peptide</th>
<th>MW of peptide–p-BQ adduct</th>
<th>Number of p-BQ molecule adducted</th>
<th>Peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid residues</td>
<td>From</td>
<td>Up to</td>
<td>From</td>
<td>Up to</td>
</tr>
<tr>
<td>1</td>
<td>52</td>
<td>73</td>
<td>2497.10</td>
<td>2605.10</td>
</tr>
<tr>
<td>2</td>
<td>94</td>
<td>106</td>
<td>1714.80</td>
<td>1822.80</td>
</tr>
<tr>
<td>3</td>
<td>206</td>
<td>212</td>
<td>1941.92</td>
<td>2049.92</td>
</tr>
<tr>
<td>4</td>
<td>238</td>
<td>274</td>
<td>1552.60</td>
<td>1660.60</td>
</tr>
<tr>
<td>5</td>
<td>360</td>
<td>372</td>
<td>854.45</td>
<td>962.45</td>
</tr>
<tr>
<td>6</td>
<td>390</td>
<td>402</td>
<td>1941.92</td>
<td>2049.92</td>
</tr>
<tr>
<td>7</td>
<td>445</td>
<td>466</td>
<td>1657.75</td>
<td>1765.75</td>
</tr>
<tr>
<td>8</td>
<td>501</td>
<td>521</td>
<td>2674.31</td>
<td>2782.31</td>
</tr>
</tbody>
</table>

Results are shown for smoker No. 5 of Fig. 1. Identical results were obtained for smoker No. 6. The detailed procedure is given in Section 2.

Table 2
MALDI-MS analysis of HSA–p-BQ adducts.

<table>
<thead>
<tr>
<th>Observed MW (Da)</th>
<th>Difference (Da)</th>
<th>Number of p-BQ in the adduct</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA</td>
<td>66,539</td>
<td>-</td>
</tr>
<tr>
<td>HSA:p-BQ(1:1)</td>
<td>66,642</td>
<td>103</td>
</tr>
<tr>
<td>HSA:p-BQ(1:3)</td>
<td>67,245</td>
<td>806</td>
</tr>
<tr>
<td>HSA:p-BQ(1:30)</td>
<td>69,131</td>
<td>5993</td>
</tr>
<tr>
<td>HSA:p-BQ(1:60)</td>
<td>70,823</td>
<td>4285</td>
</tr>
</tbody>
</table>

4 Indicates mass of intact protein before and after reaction with different molar ratios of p-BQ in the HSA–p-BQ complex.

3.3. Spectrophotometry of p-BQ-adducts of amino acids and N-acetyl amino acids

Previous results had indicated that p-BQ forms covalent adducts with Lys-rich regions of cytochrome c (Fisher et al., 2007). From the MALDI analysis it had been observed that a large number of amino acids in the HSA molecule were covalently modified by p-BQ. It is already known that amino acids react with quinone primarily through their amino groups (Kalyanaraman et al., 1987; Bittner, 2006). This reactivity has even been explored to quantify protein also (Lichti et al., 2001). In order to ascertain whether p-BQ reacted with e-amine groups of side chain Lys residues of HSA, we carried out reaction of p-BQ with the 20 common biological amino acids (Supplementary Fig. 2) and N-acetyl derivatives of Ala, Trp, Arg, His, Cys and Lys (Fig. 4) and examined the chromophores spectrophotometrically. In all the cases except cysteine and proline, formation of red or brown chromophores accompanied by absorption maxima at 350 nm is observed. In the case of cysteine, initially a colorless product is obtained, apparently due to reductive addition of the thiol group with quinone. Proline forms a colored complex with absorption maxima at around 540 nm.

3.4. Estimation of free amino groups by fluorescamine

HSA has 59 Lys residues. This means that besides the N-terminus amino group, 59 free e-amine groups of Lys are potentially

Fig. 4. Absorbance spectra of N-acetyl amino acids compared with free amino acids after treatment with p-BQ. Five hundred nano moles of amino acids were incubated with 100 nmol of p-BQ in 200 μL of 20 mM potassium phosphate buffer, pH 7.4 for 2 h at 37 °C. After incubation, 800 μL of the buffer were added to the reaction mixture and spectra taken at 200–700 nm.
Changes in the HSA bound fluorescamine fluorescence intensity before and after treatment with p-BQ; 1:1 to 1:60 indicate molar ratios of p-BQ in the HSA-p-BQ mixture. Details are given under Section 2.

available for complexation with p-BQ. With a gradual increase in the concentration of p-BQ for complexation with HSA, there will be a gradual loss of the free ε-amino groups of Lys residues. We examined the loss of free amino groups by reaction with fluorescamine. Fluorescamine reacts with the primary amino groups of proteins to produce a fluorescent product, which gives fluorescence in the range of 400–600 nm with an emission maximum at 474 nm when excited at 390 nm (Colombo et al. 2010). It has been observed that with gradual increase in the concentration of p-BQ, there is a gradual loss of fluorescamine fluorescence intensity compared to the native protein (Fig. 5). About 78% decrease in the fluorescamine fluorescence was observed for the HSA-p-BQ molar ratio of 1:60. Thus a major reduction of available ε-amino groups of Lys residues of HSA occurred after interaction with p-BQ, indicating alkylation of Lys residues of HSA by p-BQ.

3.5. Fluorescence and absorbance spectra of HSA-p-BQ complex

Interaction of p-BQ with HSA produces a reddish brown chromophore having absorption maxima (λ_{max}) at 350 nm in a time-dependent manner (Fig. 6A). Trp fluorescence of HSA is significantly quenched when HSA is preincubated with increased concentrations of p-BQ. Fig. 6B shows that the increase in the $A_{500}$ value is accompanied by concomitant decrease in the emission fluorescence (340 nm) of Trp. The fluorescence measurements were performed after dilution of the solutions using shorter pathlength cuvette to avoid inner filter effect (see Section 2). The intensity of $A_{500}$ value increased with increasing molar ratio of HSA-p-BQ from 1:1 up to 1:60 with a concomitant decrease in Trp fluorescence of about 88% (Fig. 6B). There was no observable emission of p-BQ alone under similar conditions. The Trp emission maxima of HSA in the HSA-p-BQ complex remained unaltered showing that the decrease in fluorescence intensity was mainly due to FRET. The appreciable quenching even in 1:1 molar ratio of the HSA-p-BQ complex would indicate modification of the Lys-212 residue in the vicinity of Trp-214. However, an equally plausible model would be that modification of other Lys affects structure of the protein to quench fluorescence.

3.6. Fluorescence and absorption spectra of peptide-p-BQ complex

HSA has only one Trp residue (Trp-214) and Lys-212 is present in close proximity of the Trp. To understand the effect of p-BQ on the modification of Lys-212, custom made decapeptides (see Section 2) that represent the 207–216 region of the HSA molecule containing the only Trp-214 residue were used. In the designed peptide, Nε-acetyl modification was made in Gly to block the N-terminal --NH₂ group of Gly and thereby allowing the p-BQ molecule to react with the available ε-amino group of the Lys residue. When one mole of peptide #1 (CH₃-CO-HN-Gly-Glu-Arg-Ala-Phe-Lys-Ala-Trp-Ala-Val-COOH) was incubated with 5 nmol of p-BQ, there was a marked increase of $A_{500}$ in the UV spectrum with a concomitant 70% quenching of Trp fluorescence (Fig. 7). In contrast to this, when Lys was replaced by Gly (peptide #2, CH₃-CO-HN-Gly-Glu-Arg-Ala-Phe-C/Gy-Ala-Trp-Ala-Val-COOH), there was neither any increase in $A_{500}$ nor any quenching of Trp fluorescence (Fig. 6).

3.7. MALDI-TOF-TOF analysis of the HSA-p-BQ adduct in the molar ratio of 1:1

In order to locate the Lys residue that is most susceptible for the initial formation of the p-BQ adduct, we employed MALDI-TOF-TOF method and analyzed the mass spectra obtained from the tryptic digest product of HSA-p-BQ in the molar ratio of 1:1. The MWs of the peptides expected from trypsin digestion of HSA-p-BQ in the molar ratio of 1:1. The MWs of the peptides expected from trypsin digestion of HSA with one missed cleavage was obtained from GPS software and Mascot or SWISS-PROT and 108 Da was added to the MWs to obtain the MW of the peptide-p-BQ adduct. Analysis of the MS spectra is given in Table 3. Peptides with Lys residues showing MW of peptide-p-BQ adduct have been listed. As it is not clear whether adduct formation would change the specificity of trypsin towards Lys residues, all
the Lys residues present in the peptides have been considered. The positions of Lys residues modified by p-BQ are 159, 174, 212, 262, 475, 546 or 557. Thus there is competition between the Lys residues to be modified.

3.8. Fluorescence lifetime

It is well documented that the lifetime of tryptophan is sensitive to its local environment (Engelborghs, 2001). So we have used timed-resolved fluorescence lifetime measurements of tryptophan to identify the existence of different protein conformations as well as to understand the Trp microenvironment of HSA before and after complex formation with p-BQ. The fluorescence decay raw data was fitted to a three exponential function. The average lifetime (6.55 ns) decreased gradually to 1 ns for the HSA–p-BQ complex with increased concentrations of p-BQ. This indicates substantial change in the Trp microenvironment.

3.9. CD analysis

CD spectroscopy is widely used to gain information on protein’s secondary and tertiary structure in solution. The far-UV CD (200–250 nm) of HSA shows its characteristic minima around 222 and 208 nm as expected for typical α-helical protein (Fig. 8A). With increasing molar ratio of p-BQ in the HSA–p-BQ complex, the mean residual ellipticity differed and the percentage of α-helix increased from 65.2% to 72.3% (Table 5). The CD spectrum of a protein in the near-UV region (250–320 nm) can be sensitive to certain aspects of the tertiary structure. At these wavelengths the chromophores are the aromatic amino acids and disulfide bonds. The CD signals they produce are sensitive to the overall tertiary structure of the protein. The near-UV CD (250–320 nm) spectra of HSA and HSA–p-BQ complex indicate a significant change in the tertiary environment upon complex formation (Fig. 8B). Thus covalent modification of HSA by p-BQ results in secondary and tertiary structural rearrangements of the protein.

3.10. Detection of hydrophobic region accessibility by ANS (8-anilino-1-naphthalenesulfonic acid) binding

The 8-anilino-1-naphthalenesulfonic acid (ANS) is a well known and much utilized "hydrophobic probe" for proteins (Slavik, 1982). HSA contains two hydrophobic patches located at sub-domain IIA (site 1) and sub-domain IIIA (site 2). These patches are the principal site for ligand binding (Togashi et al., 2010). Using competitive binding assay combining two fluorescence modes, three binding sites of hydrophobic molecules have been detected in HSA (Takebara et al., 2009). Changes seen in the fluorescence intensity of protein bound ANS may be attributed to alterations of accessibility of hydrophobic regions and ligand binding capacity of HSA–p-BQ complex. Fig. 9 shows that compared to native HSA, the ANS fluorescence decreases with increased molar ratio of p-BQ in the HSA–p-BQ adduct. This indicates that the solvent accessible hydrophobic regions and associated ligand binding sites of HSA become unavailable upon complexation with p-BQ. This result suggests that compared to native HSA, the ligand binding ability of HSA–p-BQ complex is altered.

3.11. Differential scanning calorimetry (DSC) studies of HSA–p-BQ adduct

To account for change in the stability of HSA after complex formation with p-BQ, we employed DSC method that directly measures the transition temperature of macromolecules. Higher melting temperature generally indicates greater stability. We have found that native HSA show three transition temperatures indicating that unfolding of HSA is not simple two-state transition. DSC
measurement of HSA–p-BQ complex shows increased melting temperature compared to pure HSA (Table 6). Table 6 summarizes the melting temperatures ($T_m$) of the first transition (peak 1) obtained for HSA and HSA–p-BQ adducts. A consistent increase in $T_m$ reveals an altered structure of HSA upon complexation with p-BQ. At higher molar ratios of p-BQ in the HSA–p-BQ complex (1:30 and 1:60), the protein forms visible aggregates probably due to formation of amyloid like structure that precipitates out of the solution accumulating within the sample port. This observation indirectly supports our CD studies. The marked increase in the $\alpha$-helix content as a function of p-BQ concentration probably makes the HSA more resistant to melting and prone to aggregation.

### Table 5
Far-UV CD analysis.

<table>
<thead>
<tr>
<th></th>
<th>HSA</th>
<th>HSA–p-BQ (1:1)</th>
<th>HSA–p-BQ (1:2)</th>
<th>HSA–p-BQ (1:4)</th>
<th>HSA–p-BQ (1:8)</th>
<th>HSA–p-BQ (1:30)</th>
<th>HSA–p-BQ (1:60)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$%$ of $\alpha$-helix</td>
<td>54.3</td>
<td>66.8 ± 0.5</td>
<td>67.6 ± 0.7</td>
<td>68.9 ± 1.4</td>
<td>70.2 ± 0.8</td>
<td>71.6 ± 1.7</td>
<td>72.3 ± 2.3</td>
</tr>
</tbody>
</table>

Percent of $\alpha$-helix was determined from the ellipticity at 222 nm according to Chen et al. (1972) (see Section 2). All experiments were done in triplicate.

* Indicates SD.

### Table 6
DSC Analysis of HSA with increasing concentrations of p-BQ in the HSA–p-BQ complex.

Data analyses were performed with Cpcalc software (Calorimetry Sciences Corp. Utah U.S.A.) and the temperature of the first transition is given in the table. The protein concentration used was ~220 μM. For 1:30 and 1:60 molar ratios, an exothermic peak was obtained beyond 100°C and thus excluded.

### Table 4
Fluorescence lifetimes of HSA as a function of increasing concentrations of p-BQ in the HSA–p-BQ complex.

<table>
<thead>
<tr>
<th></th>
<th>$r_1$ (ns)</th>
<th>$\omega_1$</th>
<th>$r_2$ (ns)</th>
<th>$\omega_2$</th>
<th>$r_3$ (ns)</th>
<th>$\omega_3$</th>
<th>$\tau_X$ (ns)</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA</td>
<td>3.6581</td>
<td>35.06</td>
<td>0.6337</td>
<td>4.72</td>
<td>7.5678</td>
<td>56.21</td>
<td>6.55</td>
<td>0.9412</td>
</tr>
<tr>
<td>HSA–p-BQ (1:1)</td>
<td>3.0606</td>
<td>35.04</td>
<td>0.45652</td>
<td>5.93</td>
<td>7.1701</td>
<td>58.13</td>
<td>6.28</td>
<td>0.7963</td>
</tr>
<tr>
<td>HSA–p-BQ (1:2)</td>
<td>2.9292</td>
<td>37.94</td>
<td>0.5581</td>
<td>8.93</td>
<td>7.6352</td>
<td>53.13</td>
<td>6.04</td>
<td>0.8846</td>
</tr>
<tr>
<td>HSA–p-BQ (1:4)</td>
<td>2.2074</td>
<td>40.82</td>
<td>0.3882</td>
<td>15.47</td>
<td>6.3364</td>
<td>43.71</td>
<td>5.24</td>
<td>0.8044</td>
</tr>
<tr>
<td>HSA–p-BQ (1:8)</td>
<td>1.7516</td>
<td>40.36</td>
<td>0.3452</td>
<td>32.01</td>
<td>5.4537</td>
<td>27.63</td>
<td>4.08</td>
<td>0.8876</td>
</tr>
<tr>
<td>HSA–p-BQ (1:10)</td>
<td>0.9072</td>
<td>55.37</td>
<td>0.3913</td>
<td>29.7</td>
<td>2.1154</td>
<td>14.94</td>
<td>1.86</td>
<td>1.1127</td>
</tr>
<tr>
<td>HSA–p-BQ (1:60)</td>
<td>0.0222</td>
<td>62.9</td>
<td>0.33809</td>
<td>25.75</td>
<td>1.4416</td>
<td>11.31</td>
<td>1.00</td>
<td>1.208</td>
</tr>
</tbody>
</table>

$^a r^2 = \langle r_1 \rangle ^2 + \langle r_2 \rangle ^2 + \langle r_3 \rangle ^2$

$^b$ The magnitude of $\chi^2$ denotes the goodness of the fit.

### Fig. 8
(A) Far-UV CD and (B) near UV-CD of HSA–p-BQ complex showing change in the secondary and tertiary structure of HSA, respectively. Details are given under Section 2.

### Fig. 9
Fluorescence of ANS bound to HSA and HSA–p-BQ complex at different molar ratios (1:1 and 1:60). Details are given under Section 2.

3.12. Ligand binding studies

Ligand binding studies were undertaken to show whether structural changes in HSA due to formation of adduct with p-BQ affects the binding capacity of HSA. Three different ligands were used...
in this study, namely, myristic acid, quercetin and paracetamol (Fig. 10A–C).

Myristic acid binding shows that the dissociation constant of fatty acid gradually increases with increasing concentration of p-BQ in the HSA–p-BQ adduct, indicating functional alteration of HSA due to structural perturbation. The dissociation constant for myristic acid increases from $(17.57 \pm 1.85) \times 10^{-6}$ M for pure HSA to $(220.67 \pm 15.5) \times 10^{-6}$ M for HSA–p-BQ 1:8 complex (Table 7, Fig. 10A). For the subsequent molar ratios (1:30 and 1:60), the dissociation constant could not be determined by fluorescence quenching due to non-detectable intensity change after ligand addition. An increase in the dissociation constant means that the HSA–fatty acid complex becomes less stable due to formation of p-BQ adduct. This might be a reason for the accumulation of lipid in the blood as observed in smokers (Craig et al., 1989).

Quercetin is weakly fluorescent in solution when excited at 400 nm and emission is observed at 550 nm, but the fluorescence intensity increases substantially when bound to protein. We have found that even at 5 μM concentration, quercetin has substantial optical density at 295 and 340 nm that may cause inner filter effect during titration by quenching method. Therefore we have employed the fluorescence anisotropy method which is suitable for measuring protein–ligand dissociation constant and much less sensitive to inner filter effects than intensity (Jameson and Mocz, 2005). The anisotropy value increased as a function of protein concentration and reached a plateau indicating saturation of binding sites (Fig. 10B). We observed that initially at low concentrations the dissociation constants decreased $(K_d)$ indicating stabilization of the complex but the values increased substantially and continuously beyond HSA–p-BQ molar ratio of 1:4 showing less affinity of HSA for quercetin (Table 7). These results indicate that in heavy smokers, where the molar ratio of HSA–p-BQ is expected to be high, the concentration of free quercetin in the blood would be high.

In the case of paracetamol binding, a greater stability of protein–drug complex (lower $K_d$) was observed for lower molar ratios of p-BQ (up to 1:8) (Table 7). This stability may have adverse effects as the drug release will be low causing less availability of the free drug concentration in the blood. On the other hand, for higher molar ratios of p-BQ (1:30 and 1:60) in the HSA–p-BQ complex, the dissociation constant decreased markedly (Table 7, Fig. 10C), indicating that under this condition the concentration of the drug in the blood may reach a very high level.

4. Discussion

In this paper we show that in smoker’s blood, p-BQ forms covalent adducts with HSA, p-BQ is not present in cigarette smoke (CS). It is apparently produced from p-BSO, a long-lived semiquinone radical present in substantial amounts in cigarette smoke (Pryor...
The dissociation constant (Kd) of HSA-p-BQ adducts in relation to the protein's secondary and tertiary structure and impairment of ligand binding properties. The formation of HSA-p-BQ adduct is apparently Michael addition of p-BQ with the ε-NH₂ groups of Lys residues. His and Arg residues of the protein are not involved in Michael addition with p-BQ.

MALDI analysis showed that when HSA was incubated with p-BQ in the molar ratio of 1:1, the p-BQ adducted preferentially with Lys-212, which is very near to the sole Trp-214. When HSA and p-BQ were incubated with increased molar ratios from 1:1 to 1:60, there was a gradual increase in the number of p-BQ molecule attached to HSA (up to 40), which was accompanied by concomitant increase in the quenching of Trp fluorescence. The quenching of the Trp-214 fluorescence indicates that the conformation of the hydrophobic binding pocket in subdomain IIA is affected (Tryndak-Lemiesz, 2004). The decrease in Trp lifetime of HSA in the HSA-p-BQ complex further reinforces our conclusion regarding change in the micro environment around the Trp residue.

Cova lent modification of Lys residues by p-BQ is accompanied by perturbation of the secondary structure of HSA as demonstrated by the far-UV CD spectra. Also the near-UV spectra indicate that after complexation with p-BQ, the tertiary structure is changed. Both these changes lead to a decrease in the number of hydrophobic pockets as observed from ANS binding studies. This result is not unexpected considering the fact that covalent modification of Lys residues by p-BQ alters the overall charge of the HSA molecule and many of the electrostatic interactions are expected to be lost resulting in changes in the secondary and tertiary conformation of the protein. Again p-BQ modification will cause a huge steric effect as shown in a model of cytochrome c-p-BQ complex, changing the intermolecular distance between the residues and causing further damage in the structure (Fisher et al., 2007).

DSC studies of HSA showed three distinct peaks indicating that the unfolding process of this protein is not a simple two-state process. The peaks might be indication of sequential melting of different domains or may be indicative of the presence of intermediates. DSC data show that upon complex formation with p-BQ, all the three transition temperatures of HSA significantly increase. A probable explanation is alteration of the structure of HSA after complexation with p-BQ, lending further support to our CD data. The DSC experiment also indicated that the ensembles at latter transition temperatures were more populated compared to free HSA, where the first transition ensemble was predominant. Thus perturbation of structure of HSA by p-BQ not only affected the native state of HSA but also influenced either the domain-domain interaction or the intermediate state. It might be concluded that covalent modification of HSA by p-BQ led to a global effect rather than simply local alterations.

Interaction with p-BQ not only alters the structure and conformation of HSA, but also its ligand binding capacity. This has been revealed by experiments with myristic acid (MA) as a representative fatty acid. The protein conformation of HSA-MA complex is very similar to that obtained by interaction of HSA with a variety of other fatty acids, including palmitic acid and oleic acid (Bhattacharya et al., 2000). So, the HSA-MA complex would display the effect of the conformational changes of HSA-fatty acid binding in general. We have indicated that the initial p-BQ molecule forms covalent adduct predominantly with Lys 212. It is also known that Trp 214 is involved in binding of MA molecule (Bojko et al., 2008). We found that the apparent dissociation constant of MA binding increased with increasing molar ratio of p-BQ in the HSA-p-BQ complex. This would indicate that binding of fatty acid with the HSA-p-BQ adduct is less stable. This might be one reason for accumulation of lipid in the smoker's blood (Craig et al., 1989) leading to atheroosclerosis.

The impairment of binding of other two ligands examined, namely quercetin and paracetamol, implied that p-BQ mediated covalent modification in HSA may affect the binding, transport and bioavailability of these two ligands in the smokers.

Quercetin (3,5,7,3',4'-penta-hydroxyflavone) is a member of naturally occurring widely distributed compounds, the flavonoids, which are found in plants, fruits, flowers and plant derived foods (Harborne and Williams, 2000). Abundant in the human diet, quercetin possesses various biological and biochemical properties including antioxidant, anti-inflammatory, antineoplastic and cardioprotective activities (Cook and Samman, 1996; Middleton et al., 2000; Dangles et al., 2001; Yang et al., 2001). As the most abundant carrier protein, HSA plays an important role in binding, transport and bioavailability of these compounds. The mechanism of formation of HSA-p-BQ adducts results in alteration of the protein's conformational and physical properties. The dissociation constant of HSA-p-BQ-quercitin complex initially decreased with increasing molar ratio of p-BQ in the HSA-p-BQ complex. This might be one reason for accumulation of lipid in the smoker's blood (Craig et al., 1989) leading to atheroosclerosis.

Table 7

<table>
<thead>
<tr>
<th>HSA</th>
<th>HSA-p-BQ (1 : 1)</th>
<th>HSA-p-BQ (1 : 2)</th>
<th>HSA-p-BQ (1 : 4)</th>
<th>HSA-p-BQ (1 : 8)</th>
<th>HSA-p-BQ (1 : 16)</th>
<th>HSA-p-BQ (1 : 32)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MYRIC</td>
<td>17.6 ± 1.8</td>
<td>50.6 ± 2.5</td>
<td>83.3 ± 4.3</td>
<td>112.7 ± 7.0</td>
<td>220.7 ± 15.5</td>
<td>319.3 ± 33.0</td>
</tr>
<tr>
<td>Quercetin</td>
<td>13.8 ± 2.9</td>
<td>16.0 ± 4.5</td>
<td>16.4 ± 4.7</td>
<td>18.9 ± 2.2</td>
<td>22.8 ± 6.2</td>
<td>53.0 ± 17.6</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>119.2 ± 4.6</td>
<td>76.8 ± 9.3</td>
<td>47.1 ± 2.7</td>
<td>44.2 ± 5.6</td>
<td>23.9 ± 2.5</td>
<td>22.93 ± 33.0</td>
</tr>
</tbody>
</table>

The HSA concentration was 0.5 μM in all cases. At least three measurements were done and standard deviations were calculated.

* Indicates no fluorescence change could be detected.

* Indicates 50.

et al., 1983; Chatterjee, 2005; Banerjee et al., 2008; Dey et al., 2010).

The results obtained with lung tissue and serum from CS-exposed guinea pigs as well as serum from human smokers indicates that after CS exposure BQ is produced in the lungs, enters the bloodstream and forms adducts with HSA. We further show that formation of HSA-p-BQ adducts results in alteration of the protein's secondary and tertiary structure and impairment of ligand binding properties. The mechanism of formation of HSA-p-BQ adduct is apparently Michael addition of p-BQ with the ε-NH₂ groups of Lys residues. His and Arg residues of the protein are not involved in Michael addition with p-BQ.

Interactions with p-BQ not only alters the structure and conformation of HSA, but also its ligand binding capacity. This has been revealed by experiments with myristic acid (MA) as a representative fatty acid. The protein conformation of HSA-MA complex is very similar to that obtained by interaction of HSA with a variety of other fatty acids, including palmitic acid and oleic acid (Bhattacharya et al., 2000). So, the HSA-MA complex would display the effect of the conformational changes of HSA-fatty acid binding in general. We have indicated that the initial p-BQ molecule forms covalent adduct predominantly with Lys 212. It is also known that Trp 214 is involved in binding of MA molecule (Bojko et al., 2008). We found that the apparent dissociation constant of MA binding increased with increasing molar ratio of p-BQ in the HSA-p-BQ complex. This might indicate that binding of fatty acid with the HSA-p-BQ adduct is less stable. This might be one reason for accumulation of lipid in the smoker's blood (Craig et al., 1989) leading to atheroosclerosis.

The impairment of binding of other two ligands examined, namely quercetin and paracetamol, implied that p-BQ mediated covalent modification in HSA may affect the binding, transport and bioavailability of these two ligands in the smokers.

Quercetin (3,5,7,3',4'-pentahydroxyflavone) is a member of naturally occurring widely distributed compounds, the flavonoids, which are found in plants, fruits, flowers and plant derived foods (Harborne and Williams, 2000). Abundant in the human diet, quercetin possesses various biological and biochemical properties including antioxidant, anti-inflammatory, antineoplastic and cardioprotective activities (Cook and Samman, 1996; Middleton et al., 2000; Dangles et al., 2001; Yang et al., 2001). As the most abundant carrier protein, HSA plays an important role in binding, transport and bioavailability of these compounds. The mechanism of formation of HSA-p-BQ adducts results in alteration of the protein's conformational and physical properties. The dissociation constant of HSA-p-BQ-quercitin complex initially decreased with increasing molar ratio of p-BQ, but increased fairly high at higher p-BQ concentration. Low dissociation constant indicates greater stability of the HSA-p-BQ-quercitin complex indicating that the level of quercitin in the blood and thereby its availability would be less. On the contrary, in heavy smokers the molar ratios of p-BQ in the HSA-p-BQ adduct would be expected to be high resulting in less binding of quercitin. Under this condition, the concentration of free quercitin in the blood would be high that might eventually cause toxicity. Quercitin is an abundant flavonoid in the human diet with numerous biological activities, which may contribute to the prevention of human disease but also may be potentially harmful. In the presence of hydrogen peroxide/peroxidases, quercitin is oxidized and covalently links to proteins with particular high affinity for HSA and thereby acts as a cytotoxic prooxidant (Metodiewa et al., 1999; Kaldas et al., 2005).

Paracetamol (N-acetyl-p-aminophenol) is a commonly used analgesic and antipyretic drug. Paracetamol has been in use as an analgesic for home medication for over 30 years and is accepted as a very effective treatment for the relief of pain and fever in adults and children. It is the most used medicine after acetylsalicylic acid in many countries as an alternative to aspirin and phenacetin (Kragh-Hansen et al., 2002; Daneshgar et al., 2009). It is known that binding to HSA controls the free, active concentration of a drug, and provides a reservoir for a long duration of action, and
ultimately affects drug absorption, metabolism, distribution, and excretion. The binding property of paracetamol showed some peculiarities. As observed with quercitin, the dissociation constant of HSA-p-BQ-paracetamol complex initially decreased at low molar ratio of p-BQ, but increased fairly high at higher p-BQ concentration. Though lower dissociation constant indicates greater stability of the HSA-paracetamol complex, but it also has physiologically adverse effect leading to less release of the amount free drug in the blood of smokers. In a light or mild smoker, the concentration of HSA-p-BQ is expected to be comparatively low. Thus a light or mild smoker may have to consume more paracetamol to get the similar clinical effect compared to a non-smoker. In heavy smokers, a higher molar ratio of p-BQ in the HSA-p-BQ-paracetamol adduct is expected to occur. Since the dissociation constant at high molar ratio of p-BQ is markedly increased, the binding and transport of the drug will be less resulting in high concentration of free paracetamol in the blood of heavy smokers. This might eventually cause hepatotoxicity of paracetamol in heavy smokers. It is known that paracetamol produces necrosis of the centrilobular cells of the liver when taken in overdose (Cook and Samman, 1996; Bessems and Vermuelem, 2001; James et al., 2003).

5. Conclusion

Results obtained with CS-exposed guinea pigs as well as human smokers indicate that after CS exposure p-BQ is produced in the lungs, enters the blood stream, and forms covalent adduct with serum albumin. p-BQ is not present in CS. It is apparently formed from p-BSQ of CS after oxidation by transition metal containing proteins in the lungs. p-BQ forms covalent adducts with p-eno groups of the lysine residues of HSA in a concentration dependent manner. This results in alterations of the secondary and tertiary structures of HSA leading to impairment of binding with ligands, including myristic acid, quercitin and paracetamol. The impairment of binding may affect the transport, distribution and metabolism of the ligands.

Conflicts of interest

The authors declare that they do not have any conflicts of interest.

Acknowledgments

We gratefully acknowledge David M. Jameson (University of Hawaii at Manoa) for critically reading the manuscript before submission; Prof. Somen Basak, Saha Institute of Nuclear Physics, Kolkata, for CD analysis; Dr. R Nagaraj, CCMB for providing some MALDI-TOF data; GlassSmithKline Pharmaceuticals Limited, India, for a gift of pure paracetamol.

Funding: This research was supported by CSIR, Government of India, grant no. 37(1368)/09-EMR-II and Juthika Research Foundation of Calcutta University. AG (PhD scholar) is a senior research fellow of CSIR; AC (PhD scholar); AD (PhD scholar) is Juthika Research Fellow; ND (PhD scholar) is a CSIR senior research fellow; IBC is INSIA Honorary Scientist. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Contributors: IBC conceived and designed major part of the experiments. RB designed all the experiments of biophysical studies. AG performed most of the experiments. AC did a part of the biophysical studies. RC, TD, KP and AD did the proteomics studies. NS did the DSC experiments. IBC and RB analyzed the data. IBC contributed reagents/materials/analysis tools and wrote the paper.
Author's personal copy