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Using lysine adducts of human serum albumin to investigate the disposition of exogenous formaldehyde in human blood

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ABSTRACT

Formaldehyde is a human carcinogen that readily binds to nucleophiles, including proteins and DNA. To investigate whether exogenous formaldehyde produces adducts in extracellular fluids, we characterized modifications to human serum albumin (HSA) following incubation of whole blood, plasma, and saliva with formaldehyde at concentrations of 1, 10 and 100 μM. The only HSA locus that showed the presence of formaldehyde modifications was Lys199. A *N*(6)-Lys adduct with added mass of 12 Da, representing a putative intramolecular crosslink, was detected in biological fluids that had been incubated with formaldehyde but not in control fluids. An adduct representing *N*(6)-Lys formylation was detected in all fluids, but levels did not increase above control values over the tested range of formaldehyde concentrations. An adduct representing *N*(6)-Lys199 acetylation was also measured in all samples. We then applied the assay to repeated samples of human plasma from 6 nonsmoking volunteer subjects (from Berkeley, CA), and single samples of serum from 15 workers exposed to airborne formaldehyde at about 1.5 ppm in a production facility and 15 control workers from Tianjin, China. Although all human plasma/serum samples contained basal levels of the products of *N*(6)-Lys formylation and acetylation, the putative crosslink product was not detected. Since the putative crosslink was observed in plasma incubated with formaldehyde at 1 μM, this suggests that the endogenous concentration of formaldehyde in serum was much lower than reported in the literature. Furthermore, concentrations of the formyl adduct were not higher in workers exposed to formaldehyde at about 1.5 ppm than in controls. Follow-up in vitro experiments with gaseous formaldehyde at 1.4 ppm detected the putative crosslink in plasma but not whole blood. This combination of results suggests that *N*(6) formylation occurs within cells with subsequent release of adducted HSA to the systemic circulation. Comparing across human samples, levels of *N*(6)-Lys199 formyl adducts were present at similar concentrations in subjects from California and China (about 1 mmol/mol HSA), but *N*(6)-Lys199 acetyl adducts were present at higher concentrations in Chinese subjects (0.34 vs. 0.13 mmol/mol HSA).

1. Introduction

Formaldehyde is an economically important chemical used to manufacture plastics, resins and intermediates as well as to preserve tissues. The International Agency for Research on Cancer (IARC) regards formaldehyde as a human carcinogen based upon increased risks of respiratory-tract cancers and myeloid leukemias in exposed workers (IARC, 2012). The designation of formaldehyde as a respiratory carcinogen is consistent with this chemical's strong

Abbreviations: AGC, automatic gain control; CV_e, coefficient of variation corresponding to the error variance; Cys, cysteine; GM, geometric mean; HSA, human serum albumin; Lys, lysine; MS, mass spectrometry; MS/MS, tandem mass spectrometry; nanoLC, nanoflow liquid chromatograph; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; RBC, red blood cells; SIC, single ion chromatogram; TCEP, tris(2 carboxyethyl)phosphine.

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electrophilicity, mutagenicity, and toxicity at the site of contact in biological systems. However, IARC's classification of formaldehyde as a potential leukemogen is controversial because leukemia can involve effects to bone-marrow cells that are remote from sites of exogenous exposure (Heck and Casanova, 2004; Swenberg et al., 2013; Zhang et al., 2009). Given its high reactivity, rapid hydration in aqueous media (to form methanediol), and endogenous production via one-carbon metabolism, the mechanism by which inhaled formaldehyde would cause leukemia is a matter of scientific importance.

Endogenous production of formaldehyde presents particular difficulties in determining the potential for exogenous formaldehyde to cause systemic effects. If inhaled formaldehyde is to enter the systemic circulation, it must be absorbed in alveolar blood. As shown in Table 1, several authors have measured endogenous formaldehyde in human blood and cultured cells (Kato et al., 2001; Ke et al., 2014; Luo et al., 2001; Nagy et al., 2004), by applying a variety of analytical methods to small samples of each matrix. Reported formaldehyde concentrations were between 1 and 18 μM in cultured cells, between 4.7 and 22 μM in whole blood, and 37 μM in plasma. These endogenous concentrations are much larger than what would be anticipated from human inhalation of formaldehyde at concentrations of a few ppm, which are the highest levels that might be encountered in occupational settings (Tang et al., 2009). Assuming continuous exposure to formaldehyde at 1 ppm (1.23 mg/m^3), a breathing rate of 1 m^3/h, and a cardiac output of 294 l/h, the maximum blood concentration of formaldehyde (assuming complete absorption in the blood) would be 0.0042 mg/l = 0.14 μM. Such a small contribution of inhaled formaldehyde to the reported endogenous blood concentrations would be consistent with results from studies of rhesus monkeys and rats, where no increase was observed in blood concentrations following 6-h inhalation of formaldehyde at either 6 or 10 ppm (Casanova et al., 1988; Kleinnijenhuis et al., 2013).

Because of its strong electrophilicity, formaldehyde readily binds to and forms crosslinks with glutathione and DNA (Lu et al., 2009; Shaham et al., 1996; Wang et al., 2009) and reacts with lysine residues to produce *N*(6)-formyllysine adducts with proteins in vitro and in vivo (Edrissi et al., 2013a, 2013b). Thus, another avenue by which inhaled formaldehyde could exert systemic effects would involve absorption by lung epithelial cells followed by release of modified glutathione, proteins and nucleic acids to the blood. In fact, immunoassays have detected putative antibodies against formaldehyde-HSA (human serum albumin) conjugates in the blood of exposed workers and smokers (Carraro et al., 1999; Ospina et al., 2011; Pala et al., 2008). Albumin is also abundant in respiratory fluids and saliva where reactions with inhaled formaldehyde would be expected (Aldini et al., 2008; Chung et al., 2013; Mellanen et al., 2001; Meurman et al., 2002; Mochca-Morales, 2000) with subsequent transfer to the blood.

Given the possibility that HSA adducts of blood and saliva could provide information regarding the disposition of inhaled formaldehyde after exposure, we characterized adducts of *N*(6)-Lys199 following in vitro reactions of formaldehyde with whole blood, plasma and saliva and also detected basal levels of *N*(6)-Lys199 acetylated adducts in the same specimens. We then measured these *N*(6)-Lys199 modifications in plasma or serum from volunteer subjects and from formaldehyde-exposed workers and control workers in Tianjin, China. (For this assay, plasma and serum specimens are essentially equivalent).

2. Materials and methods

2.1. Chemicals

Sodium borohydride ($NaBH_4$), triethylammonium bicarbonate buffer (TEAB), porcine trypsin, HSA, ammonium acetate and acetoacetanilide were from Sigma-Aldrich (St. Louis, MO). Formalin (aqueous formaldehyde 37% by weight containing 10–15% methanol as stabilizer), methanol (LCMS grade), tris(2-carboxyethyl)phosphine (TCEP), formic acid (Optima®, LCMS Grade) and acetonitrile (Optima®, LCMS grade) were from Fisher Scientific (Pittsburgh, PA). Purified water (18.2 mΩ cm resistivity at 25 °C) was prepared with a Milli-Q purification system (Millipore, Bedford, MA).

2.2. Human blood and saliva samples

Peripheral blood and saliva were collected with informed consent from groups of subjects according to human subject protocols at the participating institutions. Free formaldehyde was measured in blood that had been collected in heparin from a nonsmoking healthy Asian male. A portion of the blood specimen was immediately fractionated with Ficoll-Paque™ to obtain plasma, red blood cells (RBC), and peripheral blood mononuclear cells (PBMC). Blood for investigation of in vitro formaldehyde modifications to HSA was collected in EDTA from a nonsmoking healthy Caucasian male and immediately centrifuged at 2,000 × *g* for 3 min to separate plasma from white blood cells and RBC. This subject also provided 1 ml of saliva that was immediately centrifuged at 18,000 × *g* for 10 min using a filter (Amicon Ultra Centrifugal Filter, 0.5-ml capacity, 50 kDa MWCO, Millipore, Billerica, MA) to remove mucus and concentrate the proteins in about 20 μl.

Levels of HSA adducts from the general population were measured in plasma from 6 nonsmoking volunteer subjects in Berkeley, CA (3 males and 3 females), each of whom provided two or three peripheral blood specimens (in EDTA tubes) at intervals of three to four months. Blood samples were collected (after fasting) using EDTA tubes, which were inverted 8 times and centrifuged at 240 × *g* for 10 min, to separate plasma from buffy coats and RBC. Subsequently, buffy coats were fractionated with Ficoll-Paque to obtain PBMC and granulocytes/RBC by centrifuging at 380 × *g* for 40 min.

Blood samples were also obtained in 2012 from 15 formaldehyde-exposed (4 males and 11 females) and 15 unexposed workers (5 males and 10 females) in Tianjin, China. (All subjects were nonsmokers). Exposed subjects worked in a formaldehyde-

Table 1
Concentrations of endogenous formaldehyde reported in human blood and cultured human cells.

Biological matrix	Method	Formaldehyde conc. (μM)	N	Notes	Reference
Whole blood	GC–MS	87 ± 4.7	6	Mean ± S.D.	(Heck et al., 1985)
Whole blood	LC–MS	22	1		(Nagy et al., 2004)
Plasma	Derivatization + LC-fluorescence	37 (29–45)	2	Median (Range)	(Luo et al., 2001)
Nasal epithelial cells	Derivatization + fluorescence	3	2	At LOD	(Neuss et al., 2010)
Cancer cells	SIFTCI-MS	1.3 (0–4)	6	Median (Range)	(Kato et al., 2001)
HeLa cells	Derivatization + spectrophometry	18	1		(Ke et al., 2014)

Legend: GC, gas chromatography; LC, liquid chromatography; LOD, limit of detection; MS, mass spectrometry; SIFTCI, selected-ion-flow-tube chemical ionization