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DNA damage in internal organs after cutaneous exposure to sulphur mustard

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ABSTRACT

Sulphur mustard (SM) is a chemical warfare agent that attacks mainly skin, eye and lungs. Due to its lipophilic properties, SM is also able to diffuse through the skin and reach internal organs. DNA represents one of the most critical molecular targets of this powerful alkylating agent which modifies DNA structure by forming monoadducts and biadducts. These DNA lesions are involved in the acute toxicity of SM as well as its long-term carcinogenicity. In the present work we studied the formation and persistence of guanine and adenine monoadducts and guanine biadducts in the DNA of brain, lungs, kidneys, spleen, and liver of SKH-1 mice cutaneously exposed to 2, 6 and 60 mg/kg of SM. SM-DNA adducts were detected in all studied organs, except in liver at the two lowest doses. Brain and lungs were the organs with the highest level of SM-DNA adducts, followed by kidney, spleen and liver. Monitoring the level of adducts for three weeks after cutaneous exposure showed that the lifetime of adducts were not the same in all organs, lungs being the organ with the longest persistence. Diffusion from skin to internal organs was much more efficient at the highest compared to the lowest dose investigated as the result of the loss of the skin barrier function. These data provide novel information on the distribution of SM in tissues following cutaneous exposures and indicate that brain is an important target.

Introduction

Sulphur mustard (bis-(2-chloroethyl) sulphide, SM) is a well known chemical warfare agent. It was first used during World War I and more recently in the Iran/Iraq conflict causing more than 100,000 Iranian casualties (Balali-Mood and Hefazi, 2006). Because stocks of SM are available, it remains a military threat (Smith et al., 1995). Moreover, SM is quite simple to synthesize and could be used for terrorist attacks against the general population (Wattana and Bey, 2009). Skin, eyes and lungs represent the three main target organs of SM. Immediate as well as long term effects have been documented in human exposed to SM. One of the most characteristic acute effects of SM is the induction of severe skin burns by the formation of blisters which do not properly heal. (Balali-Mood and Hefazi, 2005; Ghanei et al., 2010; Momeni et al., 1992; Naraghi et al., 2005) SM is consequently classified as a vesicant. In addition to the formation of cutaneous lesions, skin is of interest in SM toxicity because it may be considered as an entry to internal organs. It was reported that SM deposited on skin does not remain there (Chilcott et al., 2000; Cullumbine, 1946). Only 20% of the applied dose is absorbed, the remaining 80% being evaporated. Less than 20% of absorbed SM reacts with biomolecules in skin and more than 80% enters in the blood flow and diffuses towards internal organs or is eliminated in biological fluids. It is thus expected that cutaneous exposure to SM leads to damage in internal organs.

DNA damage has been shown to play an important role in the deleterious effects of SM. In particular, genotoxic insult was proposed to be a major event in the massive cell death induced by acute exposure to SM and responsible for the degradation of target organs (Debiak et al., 2009; Kehe et al., 2009; Papirmeister et al., 1985). In addition, SM is recognized as an efficient carcinogen in humans in relationship with its DNA damaging properties (Easton et al., 1988; Emadi et al., 2008; Inada et al., 1978). Interestingly, reports have been made on induction of internal cancers in humans exposed to SM (Easton et al., 1988; Yamakido et al., 1996). Investigating the formation of SM-DNA adducts in internal organs after cutaneous exposure appears as an important issue to better understand SM toxicity.

From a mechanistic point of view, SM is a powerful alkylating agent that damages DNA mostly through the formation of adducts. These involve positions N7 of guanine (N7-hydroxyethylthioethyl-guanine, HETE-N7Gua) and N3 of adenine (N3-hydroxyethylthioethyl-adenine, HETE-N3Ade). In addition crosslinks between two guanines through the N7 atom (bis(N7-guanine)-ethylthioethyl, N7Gua-ETE-N7Gua) are also produced. These adducts have been well characterized

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in isolated DNA (Brookes and Lawley, 1960, 1961, 1963; Fidder et al., 1994; Ludlum et al., 1994) and to some extent in cells (Fidder et al., 1994; Ludlum et al., 1994). I*n vivo* data have also been gathered for HETE-N7Gua (van der Schans et al., 2004). A characteristic of SM-DNA adducts is the instability of the N-glycosidic bonds that they induce. The resulting depurination reaction leads to release of a free modified base and formation of an abasic site in DNA like for most N7 guanine adducts (Boysen et al., 2009).

Using a recently developed high performance liquid chromatography-tandem mass spectrometry assay (HPLC-MS/MS) (Batal et al., 2013a), we described in previous work for the first time the formation and persistence of SM-DNA adducts in skin of SKH-1 mice (Batal et al., 2013b). Using samples collected from the same animals, we now report information on the level of SM-DNA adducts over a period of three weeks following exposure to 2, 6 and 60 mg/kg of SM in several internal organs: brain, lungs, kidneys, spleen and liver. These data on DNA damage are interesting for the understanding of the putative onset of carcinogenic processes and SM-DNA adducts may also be considered as biomarkers of the internal exposure to SM in different organs.

Material and Methods

Caution

SM is a carcinogenic and cytotoxic agent. It should be handled carefully in a fume hood with adequate protection by experienced personnel.

Chemicals and enzymes

SM was obtained from the French Single National Small-Scale Facility (DGA Maîtrise NRBC, Vert-Le-Petit, France) and was > 95 % pure. All enzymes and chemicals were obtained from Sigma (St. Louis, MO, USA). Exceptions were protease purchased from Qiagen and phosphodiesterase I from Worthington (Lakewood, NJ, USA). HPLC-grade methanol was from Carlo Erba (Milano, Italy) and acetonitrile from Merck (Darmstadt, Germany).

Animals

Male, euthymic and hairless SKH-1 mice (Crl: SKH1-*hr*) obtained from Charles River Laboratories (L'Arbresle, France) were 4–6 weeks of age on the date of delivery. Animals were housed and acclimatized for 7 days before experiment in a controlled environment with food and water *ad libitum*. Experiments were in accordance with the applicable French and European Community regulations. Study protocols were approved by the CRSSA-IRBA Institutional Animal Care and Research Advisory Committee.

SM exposure

Before exposure, animals were anesthetized by an intraperitoneal injection of a mixture of diazepam (3.75 mg/kg) and ketamine hydrochloride (33.30 mg/kg). Mice were then randomly assigned to treatment groups. Five or 6 mice were included in exposed groups and 4 mice in control group. For pain relief, buprenorphin (0.05 mg/kg) was delivered by a subcutaneous injection during the first 3 days. SM, diluted in two μL of dichloromethane, was applied topically on a circular zone of 0.28 cm^2 before hand ink-marking on the dorsal-lumbar region of the animal centred on the body axis (Dorandeu et al., 2011). Animals were exposed to 2, 6 and 60 mg/kg of SM. Control animals were treated following the same protocol by replacing SM solution by pure dichloromethane. Four hour after SM exposure, exposed skin site was then gently wiped with a fresh solution of 0.8 % sodium hypochlorite using natural sponges to perform skin decontamination as recommended by Suchard (Suchard, 2011) to remove any residual contact hazard. Mice were euthanized by injection of an overdose of sodium pentobarbital (*ca.* 100 mg/kg, i.p.) 6 hours, 1, 3, 7, 14 and 21 days after the beginning of the exposure. Brain, lungs, kidneys, spleen and liver were collected and frozen in liquid nitrogen. Samples were stored at −80 °C until use.

Extraction, digestion and HPLC-MS/MS analyses of DNA

DNA extraction from the different organs was achieved as previously described (Ravanat et al., 2002). The level of N7Gua-ETE-N7Gua, HETE-N7Gua and HETE-N3Ade was then determined by using a method recently developed in our group (Batal et al., 2013a). Extracted DNA was enzymatically hydrolyzed into nucleosides by using a cocktail of endonucleases, phosphodiesterases and phosphatase. A thermal treatment was applied to achieve quantitative depurination of SM-DNA adducts. Samples were injected in a reverse phase HPLC system (Agilent series 1100) coupled to an API 3000 triple quadrupolar mass spectrometer (PerkinElmer/SCIEX, Thornhill, Canada). SM-DNA adducts were detected on the basis of specific fragmentation reactions. A reverse phase C18 column was used with a gradient of acetonitrile in 2 mM ammonium formate (Batal et al., 2013a). The amount of normal nucleosides was quantified by a UV detector. Quantification of DNA lesions and normal nucleosides was performed by external calibration using calibrated solutions of authentic standards. Results are expressed as the number of adduct per million bases.

Statistical analyses

The data were analyzed using Statistica 8.0 (ChemBioOffice®, Cambridge, MA). Results were expressed as mean ± SEM (control: n = 4; treated: n = 5 or 6). For comparison of dose and time effect on SM-DNA adducts frequency, data were analyzed using the U test of Mann–Whitney. Statistical significance was considered to be reached for p values lower than 0.05. Rates of elimination were calculated on the basis of first-order kinetics. The disappearance rate constants (k) were determined by linear regression of the plot of Log(A0/A) versus time, where A was the level of DNA adducts. Statistical significance of first-order kinetics was validated by the calculation of the Pearson's coefficient. Then, t-value was calculated and used for Student's test with n-2 freedom degrees. Standard errors of the linear regression were calculated. Reported rates of removal of adducts correspond to data with p values below 0.001. In order to provide more biological meaningful values, first order rate constants were converted and presented as half-lifetimes (Log(2)/k),

Results

DNA damage to internal organs early after SM skin exposure

As soon as the first time of animal sacrifice, namely 2 h after the end of the 4 hour-exposure to SM (referred to as "6 h post-exposure"), SM-DNA adducts were detected in all organs and for all doses, with exception of liver for mice exposed to 2 and 6 mg/kg and the adduct HETE-N3Ade in spleen of mice treated to 2 mg/kg (Table 1). No adduct was detected in the organs of untreated mice. For all doses, the level of adducts was similar in brain and lungs for 2 and 6 mg/kg. At 60 mg/kg, the amount of DNA adducts was almost 2-fold larger in brain but this difference was not statistically significant. The amount of adducts was lower in kidneys and spleen and roughly one order of magnitude lower in liver (at 60 mg/kg). In all organs and for all doses, the three adducts were produced in the following decreasing order of frequency: HETE-N7Gua > N7Gua-ETE-N7Gua > HETE-N3Ade. Dose dependent effect was observed for all adducts but was not linear. Proportionality in the level of SM-DNA adducts was observed when the two lowest doses were compared. In contrast, an increase by a factor much larger than the expect 10 was observed when increasing the applied dose from 6 to 60 mg/kg depending of organs and adducts studied. The value of this ratio was 42, 23, 252 and 29 for HETE-