Retrospective Detection of Sulfur Mustard Exposure by Mass Spectrometric Analysis of Adducts to Albumin and Hemoglobin: An In Vivo Study

TNO Defence, Security and Safety, P.O. Box 45, 2280 AA Rijswijk, The Netherlands

Abstract

The persistence in rats of sulfur mustard adducts to albumin and hemoglobin was studied in vivo after exposure (intravenously; 0.3 mg/kg; approximately 0.1 LD₅₀) of rats to sulfur mustard. The albumin adduct (S-HETE)Cys-Pro-Tyr was detectable up to 7 days after the exposure, while the adduct to the N-terminal valine in hemoglobin was still detected after 28 days. The decrease in adduct levels corresponded well with the half-life time of albumin in rats and with the lifetime of the rat erythrocyte. Remarkably, the N-terminal valine adduct to hemoglobin increased during the first three days, which implies that there is still free sulfur mustard present during that time. In contrast, the corresponding albumin adduct levels did not increase during this time period. The free sulfur mustard might have accumulated in the erythrocyte cell membrane.

Experimental

Introduction

Within the framework of our continuing program towards the development of methods for diagnosis and dosimetry of exposure to chemical warfare agents, we have reported several assays for sulfur mustard that are based on adducts that the agent forms in blood and skin (1-6). The main advantage of detection of adducts of sulfur mustard to proteins over those to DNA is the expected much longer half-life of protein adducts (7). It is expected that adducts to proteins have life-spans, varying from several weeks to months (8,9). Moreover, detection is supposedly also more sensitive in the case of single, protracted, and intermittent exposure to sulfur mustard at low concentrations, because the protein adducts will accumulate.

Currently, two sulfur mustard protein adduct-based methods exist that have recently been significantly improved (3,4). The albumin-tripeptide assay is based on the fact that pronase digestion of albumin alkylated by sulfur mustard results in the formation of (S-HETE)Cys-Pro-Phe, which can be analyzed by liquid chromatography–tandem mass spectrometry (LC–MS–MS) (10). The modified Edman procedure is based on the selective cleavage of the N-terminal valine adduct to human hemoglobin, followed by gas chromatography (GC)–MS analysis of the derivatized sulfur mustard adduct (3,11). Interestingly, the albumin-tripeptide method was recently advantageously applied to blood samples from a victim that had been accidentally exposed to sulfur mustard (12). We report here the applicability of both the albumin-tripeptide assay and the modified Edman procedure towards in vivo exposures of rats, in order to study the persistence of the particular protein adducts.

Instrumentation

Experiments were conducted by LC–electrospray–MS–MS on a quadrupole-time-of-flight (Q–TOF) hybrid instrument equipped with a standard Z-spray electrospray interface (Waters, Altrincham, U.K.) and an Alliance (type 2690 LC, Waters, Milford, MA). The chromatographic hardware consisted of a pre-column splitter (type Accurate; LC Packings, Amsterdam, The Netherlands), a six-port valve (Valco, Schenkon, Switzerland), a 50-μL injection loop, and a PepMap C₁₈ column (3μm, 15 cm x 1 mm, LC Packings).

LC system conditions

Eluent A: 0.2% formic acid in water. Eluent B: 0.2% formic acid in acetonitrile. At time 0: 100% A/0% B, 0.1 mL/min flow. At 5 min: 100% A/0% B, 0.6 mL/min flow. At 50 min: 30% A/70% B, 0.6 mL/min flow. The flow of 0.6 mL/min was split before the column to 35 μL/min.

Triple Q–MS conditions

Transitions of the m/z 486 and m/z 494 protonated molecular ions of (S-HETE)Cys-Pro-Tyr and (S-HETE-d₈)Cys-Pro-Tyr, respectively, to the most intense fragments m/z 105
was incubated with sulfur mustard-d8 in acetonitrile (100 μM; 0.3 mg sulfur mustard/kg (i.v.; approximately 0.1 LD50)). Sulfur mustard was diluted to a concentration of 6 mg/mL in 2-MeTHF (2500 rpm, 5 min). Splitless time: 1 min. MSD transfer line: 240°C. MS quad: 130°C. MS source: 160°C. EMV: 1576 V. MSD mode: SIM, m/z 564, m/z 572. Dwell time: 80 ms.

Determination of persistence of sulfur mustard adducts in rats exposed to sulfur mustard (i.v.)

Male Wistar WU rats (approximately 300 g) were purchased from Harlan, The Netherlands. The animals were allowed to eat and drink ad libitum. They were allowed to acclimatize to their new environment for at least one week before they were used in any experiment. The protocols for animal experiments were approved by the TNO Committee on Animal Care and Use. Rats (three animals per time point) were exposed to a dose of 0.3 mg sulfur mustard/kg (i.v.; approximately 0.1 LD50). Sulfur mustard was diluted to a concentration of 6 mg/mL in 2-propanol. Just before injecting the rats, the sulfur mustard solution was diluted with saline to give a concentration of 0.3 mg/mL. After anesthesia with Dormicum/Hypnorm, two rats at a time were injected with this freshly prepared solution (1 mL/kg, i.v.) in the penis vein.

At established time points (10 min, 1 h, 6 h, 1, 2, 3, 7, and 28 days) after exposure, animals were killed by decapitation. Blood (approximately 7 mL/rat) was collected in heparinized tubes and centrifuged (2500 rpm, 5 min) to separate plasma from erythrocytes. The plasma samples were stored at −20°C until further work-up. The erythrocytes were washed three times (2500 rpm, 5 min) with phosphate buffered saline (PBS) (7 mL) and subsequently stored at −20°C.

Preparation of internal standards for analysis of albumin and hemoglobin adducts

Rat blood (10 mL, heparinized) from unexposed animals was incubated with sulfur mustard-d8 in acetonitrile (100 μM; final acetonitrile concentration 1%) for 2 h at 37°C. Next, plasma was separated from erythrocytes by centrifugation (2500 rpm, 5 min). The plasma was stored at −20°C and was used as internal standard for analysis of the plasma samples generated throughout the animal experiments. The erythrocytes were washed three times (2500 rpm, 5 min) with PBS and hemolized in water. Subsequently, globin was isolated according to the procedure described by Bailey et al. (13) and used as internal standard for analysis of the globin samples generated throughout the animal experiments.

Work-up of rat plasma samples

To rat plasma (0.5 mL) was added plasma (25 μL), isolated from rat blood exposed to 100μM sulfur mustard-d8. This mixture was diluted with buffer A (2 mL, 50mM KH2PO4, pH 7.0) and filtered using a 0.45-μm filter disc in order to remove solid particles. Next, the sample was applied on a Hitrap Blue Sepharose column (Amersham Biosciences, 1 mL, capacity 20-mg human serum albumin/ml gel) and washed with buffer A (12 mL). Next, albumin was eluted using buffer B (50mM KH2PO4, 1.5M KCl, pH 7.0). The entire wash and elution steps were monitored with a UV lamp at 280 nm. UV-positive material was collected, resulting in a total volume of 3 mL. These 3-mL samples were desalted using a PD-10 desalting column (Amersham Biosciences). The PD-10 column was equilibrated using a solution of NH4HCO3 (50mM, 25 mL). Next, the sample consisting of buffer B (3 mL) and albumin was applied. The albumin was eluted using 3 mL of NH4HCO3 solution (50mM).

Pronase digestion of rat albumin, followed by LC–MS–MS analysis

Part of the previously mentioned solution (0.75 mL, containing maximal 4.8 mg albumin) was digested using pronase (100 μL, 10 mg/mL in 50mM NH4HCO3) for 2 h at 37°C. After 2 h, the mixture was filtered using a 10 kDa MW-cutoff filter. The filtrate was analyzed using Q-TOF LC–MS and LC-MS-MS for the presence of (S-HETE)Cys-Pro-Tyr and its deuterated analogue.

Analysis of rat globin samples according to the procedure for modified Edman degradation of globin

Globin (20 mg), isolated from rat blood, was dissolved in formamide (2 mL). Subsequently, a solution of globin isolated from rat blood exposed to sulfur mustard-d8 (100μM) in formamide (20 mg/mL; 50 μL) was added. Next, pyridine (8 μL) and pentafluorophenyl isothiocyanate (8 μL) were added and the mixture was incubated at 60°C in a heating block for 2 h. After cooling to room temperature, the mixture was extracted with toluene (3 x 1 mL) by means of mixing the toluene with the formamide solution using a Vortex (30 s) and centrifuging in a Jüran RC 10.10 centrifugal evaporator for 2 min (1200 rpm). Next, the samples were frozen in liquid nitrogen in order to achieve a better separation of the two layers. The toluene layers were combined, washed with water (2 x 0.5 mL), aqueous Na2CO3 (0.1M, 0.5 mL), and water (0.5 mL). The organic layer was dried (MgSO4), evaporated to dryness using the centrifugal evaporator, and dissolved in toluene (100 μL). Next, a Florisil cartridge was conditioned with methanol/dichloromethane (1:9, v/v; 2 mL) and dichloromethane (2 mL). The toluene solution was applied on the cartridge, which was subsequently washed with dichloromethane (2 mL) and methanol/dichloromethane (1:9, v/v; 1 mL). The thiolydantoin was eluted with methanol/dichloromethane (1:9, v/v; 1.5 mL). The latter eluate was evaporated to dryness and dissolved in toluene (100 μL). To this solution, heptafluorobutyryl imidazole (10 μL) was added, and the mixture was heated at 60°C for 30 min. After cooling, the reaction mixture was washed with water (2 x 100 μL), aqueous Na2CO3 (0.1M, 100 μL), and finally with water (100 μL). The toluene layer was dried (MgSO4), concentrated to 30 μL, and analyzed by GC–MS.
Results

Persistence of albumin adducts
In order to obtain information about the persistence of the albumin-sulfur mustard adduct, rats were exposed to the agent. We prefer to use rat for these studies because the amino acid sequence of rat albumin is known. Furthermore, it has been reported that the modified tripeptide Cys-Pro-Tyr results from rat albumin modified at Cys-34 by the food-borne carcinogen 2-amino-3-methylimidazo[4,5-f]quinoline (IQ, 14), after pronase treatment. Experiments in which rat blood was exposed in vitro to sulfur mustard, showed that the major product was (S-HETE)Cys-Pro-Tyr (see Figure 1 for tandem MS spectrum), and that the level of alkylation of the free cysteine residue in rat albumin was approximately 5% of total alkylation of albumin (results not given, obtained by using [14C]-labeled sulfur mustard). ESI-MS-MS analyses were performed in an analogous way as for (S-HETE)Cys-Pro-Phe (i.e., by selecting the charged molecular ion and monitoring the highly selective m/z 105 fragment).

The animal study was performed as follows. For each time point to be studied, 3 rats were used. A dose of 0.3 mg/kg (approximately 0.1 LD₅₀) sulfur mustard was administrated (i.v.) to the animals, and blood was collected after 10 min, 1 h, 6 h, 1, 2, 3, 7, and 28 days after exposure. At the indicated time points, blood was collected (approximately 7 mL per rat). Plasma was separated from the erythrocytes by centrifugation and after addition of a well-defined amount of internal standard (i.e., plasma from rat blood that had been exposed to 100µM sulfur mustard-d₈), albumin was isolated by affinity chromatography on Blue Sepharose cartridges, according to Standard Operating Procedure. After treatment with pronase, the resulting digests were analyzed qualitatively by means of LC–MS–MS on a Q-TOF instrument, with acquisition of full MS–MS spectra.

Subsequently, the samples were analyzed by means of LC–MS–MS on a triple-Q instrument for more sensitive analysis using the multiple reaction monitoring mode. A representative analytical run is given in Figure 2; the tandem MS spectrum of in vivo formed (S-HETE)Cys-Pro-Tyr was identical to that obtained after in vitro exposure to rat blood (Figure 1). Significant amounts of the tripeptide could be observed, which rapidly decreased in time (half-life of sulfur mustard-modified albumin: 2 days). For the time-course of the albumin adduct level, see Table I and Figure 3.

Persistence of N-terminal valine adduct in hemoglobin

The N-terminal valine adduct levels were determined in the corresponding erythrocyte samples according to the modified Edman procedure (3), using our isotope dilution quantification method. A representative analytical run is shown in Figure 4. The results of the various analyses are given in Table II and Figure 5.

Discussion

From in vitro experiments with rat blood and [14C]-labeled...
sulfur mustard, we learned that the free cysteine-34 residue in rat albumin is also susceptible to alkylation by sulfur mustard, similar to human albumin. Thus, a tripeptide (S-HETE)Cys-Pro-Tyr was released after pronase digestion, and the level of alkylation at the particular cysteine residue was approximately 5%. On the basis of these results, we thought that the rat is a good animal model for studying the persistence of the adduct.

Rats were exposed (i.v., 0.3 mg/kg) to sulfur mustard, and at certain time points the animals were killed and their blood was collected. After addition of internal standard, albumin was isolated from the plasma by affinity chromatography. Subsequently, the albumin samples were treated with pronase and the digests were analyzed by means of LC–MS–MS. Initially, an increase in adduct level could be observed. Subsequently, the adduct level decreased rapidly. The observed half-life of sulfur mustard alkylated albumin was 2 days, which is in accordance with literature values for albumin adducts [1–3 days (15)].

The albumin adduct was no longer detectable at 7 days after the exposure. In contrast to rats, the half-life of albumin is much higher (20–25 days), suggesting that the adduct might be detectable for a longer period of time after exposure.

The corresponding globin samples were analyzed for the presence of adducts to the N-terminal valine residues. Remarkably, the N-terminal valine adduct level clearly increased during the first three days, which suggests that there is still free sulfur mustard present during that time, which causes ac-
cumulating damage. Interestingly, Langenberg et al. (16,17) have observed a long half-life of sulfur mustard in blood after intravenous exposure of hairless guinea pigs to sulfur mustard. The slow elimination of sulfur mustard was observed by Maisonneuve et al. (18), after i.v. administration of the agent to rats. Accumulation of albumin adducts was less pronounced and only observed during the first hours after the exposure. This can only partly be explained by the much faster elimination of the albumin adduct, in comparison with the hemoglobin adduct. Apparently, sulfur mustard accumulates in the cell membrane of the erythrocytes, from which it is slowly released, thereby forming adducts with intracellular hemoglobin. The N-terminal valine adduct level decreased more or less linearly, in accordance with the life-time of the erythrocyte of the rat (reported life-time of rat erythrocyte 65 days [19]). The adduct to the N-terminal valine could still be detected after 28 days. Further comparison of the two time-courses (i.e., from hemoglobin adduct and albumin adduct levels) shows that the rat the hemoglobin adduct is far more persistent than the albumin adduct, which is in agreement with the life-time of the rat erythrocyte and the half-life of rat albumin.

In conclusion, after exposure (i.v.) of rats to sulfur mustard, the albumin adduct (5-HETE)Cys-Pro-Tyr) was detectable 7 days after the exposure, and the adduct to the N-terminal valine could still be detected after 28 days. The results clearly show that both assays can be advantageously employed for determination of sulfur mustard exposures.

Acknowledgment

The work described in this paper was supported by the U.S. Army Medical Research and Materiel Command under contract DAMD17-02-2-0012.

References

