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Comparison of styrene-7,8-oxide adducts formed via reaction with cysteine, N-terminal valine and carboxylic acid residues in human, mouse and rat hemoglobin

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Abstract

The reactive metabolite of styrene, styrene-7,8-oxide (SO), reacts with a variety of nucleophilic sites in hemoglobin (Hb) to form SO-Hb adducts. Following the in vitro incubation of SO with blood from humans, NMRI mice and Sprague–Dawley rats, the second-order reaction rate constants were determined for the reaction of SO with cysteine (through both the α- and β-carbons of SO), N-terminal valine (through the β-carbon of SO), and carboxylic acid (presumably through both the α- and β-carbons of SO) residues in Hb. The rate constants for cysteine adducts vary dramatically between species [2.04, 10.7, 133 L (mol Hb)$^{-1}$ h^{-1} (α binding) for humans, mice and rats, respectively] and [0.078, 2.16, 20.4 L (mol Hb)$^{-1}$ h^{-1} (β binding), respectively]. The considerably higher rate of reaction with

Abbreviations: AUC, area under the curve; GC, gas chromatography; Hb, hemoglobin; MS, mass spectrometry; PE, phenylethanol; PFB, pentafluorobenzoyl-; S.E., standard error; SG, styrene glycol; SIM, selected ion monitoring; SO, styrene-7,8-oxide; SO-cys, SO-val, SO-COOH, Hb adducts of SO due to binding at cysteine, N-terminal valine, and carboxylic acid residues, respectively.

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cysteine in rat Hb probably reflects the presence of an additional cysteine residue at position β125. Although the rate constants for valine adducts (1.82, 0.80, 0.29 L (mol Hb)$^{-1}$ h^{-1}, respectively) and COOH adducts (3.55, 1.94, 2.37 L (mol Hb)$^{-1}$ h^{-1}, respectively) are much more consistent, the inter-species differences are statistically significant for the reaction of SO with the N-terminal valine of Hb. Following the i.p. administration of styrene to mice and styrene and SO to rats, the levels of adducts at each of these sites were used in conjunction with the calculated rate constants to predict the integrated blood doses of SO. While the SO doses predicted from cysteine and valine adducts were very similar, that based upon COOH-binding was significantly different, presumably due to the instability of SO-COOH adducts. This research affirms the use of both cysteine and valine adducts, but not carboxylic acid adducts, as biomarkers of exposure to styrene and SO. © 1997 Elsevier Science Ireland Ltd.

Keywords: Styrene; Styrene-7,8-oxide; Raney nickel; Modified Edman degradation; Hemoglobin adducts; Biomarkers

1. Introduction

Styrene-7,8-oxide (SO) is the primary metabolite of styrene, an important chemical used worldwide in the production of plastics and resins [1]. SO readily reacts with nucleophilic sites on biological macromolecules such as DNA and proteins (reviewed in [2]), and has been found to be both mutagenic (reviewed in [3]) and carcinogenic in animals [4–6]. SO is further metabolized by glutathione *S*-transferases (in rodents) and epoxide hydrolases, with most of the dose excreted in urine as mandelic acid and phenylglyoxylic acid [7].

In an attempt to better define the dose-response relationship between exposure to styrene or SO and their potential to cause genetic damage, protein adducts have been proposed as specific biomarkers of longer-term exposures due to the long life span of proteins such as hemoglobin (Hb) (i.e. 120 days in humans, corresponding to the life span of the red blood cell). Since protein adducts are not repaired (although some are chemically unstable) and protein turnover tends to follow simple zero or first-order kinetics, protein adduct levels are proportional to the average concentration of the metabolite in blood (or other tissues) and, if stable, can be used to estimate the time-integrated dose of the reactive intermediate of interest [8].

SO is capable of binding to a variety of nucleophilic sites on proteins, such as the sulfhydryl group of cysteine, the amino group of lysine, the histidine imidazole, the tyrosine hydroxyl group, the glutamic acid, aspartic acid and C-terminal carboxylic acid groups, and the N-terminus [2]. Three site-specific techniques for the analysis of SO-protein adducts have been developed and applied to investigations of both rodents and styrene-exposed workers. The sites analyzed are the N-terminal valine of Hb, following a modified Edman degradation [9–15]; the carboxylic acid

residues in Hb and albumin, following hydrolysis to styrene glycol (SG) [16–19]; and the cysteine residues in Hb and albumin, following reaction with the reductive catalyst Raney nickel [19–22].

Given a stable adduct, analysis of any of these site-specific SO adducts, combined with their corresponding second-order reaction rate constants, should be useful for estimating the blood dose of SO in both rodent and human studies. Although all three assays have been used in biomonitoring studies of reinforced-plastics workers, the results have been mixed. Christakopoulos et al. [11] found statistically significant increases in Hb adducts of N-terminal valine with respect to urinary metabolite levels in seven workers (estimated mean exposure of 300 mg/m^3), while Brenner et al. [10] found no significant difference in Hb adducts from 14 workers (estimated exposure of 4–190 mg/m^3 of styrene) compared to eight controls and Severi et al. [12] detected no such adducts (detection limit = 10 pmol/g globin) in 52 workers (estimated exposure 2.2–110 mg/m^3 of styrene). Regarding adducts of carboxylic acid residues, Sepai et al. [18] were not able to detect adducts and Yeowell-O'Connell et al. [19] were unable to detect a significant correlation between styrene exposure and SO-COOH adducts. With respect to cysteine adducts, Yeowell-O'Connell et al. [19] demonstrated an exposure-dependent increase in albumin, but not Hb adducts in 48 workers exposed to estimated levels of 0.9–235 mg/m^3 of styrene, while Fustinoni et al. [22] reported significant increases in both albumin and Hb adducts in comparison to the corresponding levels of urinary metabolites in 22 workers (estimated mean styrene exposure of 100 mg/m^3). Such inconsistent results make it difficult to evaluate the relative utility of each of these techniques for the analysis of SO-Hb adducts, and, heretofore no direct comparisons have been made.

The primary objective of this study was to compare the predicted blood doses of SO based upon the analysis of SO-cys, SO-val and SO-COOH Hb adducts following the i.p. administration of styrene to NMRI mice and styrene or SO to Sprague–Dawley rats. An underlying objective involved the estimation of the in vitro first-order elimination rates of SO from human, mouse and rat blood and the calculation of site-specific second-order reaction rate constants for the reaction of SO with cysteine, N-terminal valine and carboxylic acid residues in Hb from the three species.

2. Methods

2.1. Reagents and internal standards

Please note that styrene, SO and the derivatization reagents are considered toxic and should only be handled in a hood. All reagents were the same as those reported in Rappaport et al. [21], Severi et al. [12] and Pauwels et al. [14].

The protein-bound internal standard used in the analysis of carboxylic acid and cysteine-bound adducts, 4-methyl-SO-Hb, was the same as that described in Rappaport et al. [21]. The level of adducts in the modified protein was determined by

repeatedly assaying the samples following either base hydrolysis (to release 4-methyl-SG) or reaction with Raney nickel (to release 4-methyl-2-phenylethanol (4-methyl-2-PE)) as described in Yeowell-O'Connell et al. [19]. The mean level of carboxylic acid-bound 4-methyl-SO was found to be 8.12 (S.E. = 0.23, $n = 8$) nmol of 4-methyl-SG/mg 4-methyl-SO-Hb and the mean level of cysteine-bound 4-methyl-SO was found to be 0.397 (S.E. = 0.024, $n = 6$) nmol of 4-methyl-2-PE/mg 4-methyl-SO-Hb. The adduct yield of 4-methyl-2-PE from modified Hb was lower than that reported previously [21] due to the additional base hydrolysis step used to release COOH-bound SO [19].

The internal standard used in the analysis of N-terminal valine adducts, [$^2H_8$]SO-Hb, was prepared as described in Severi et al. [12] and, following acid hydrolysis and the modified Edman degradation, was found to be alkylated with 1.73 nmol of *N*-(2-hydroxy-2-[$^2H_8$]phenylethyl)valine/mg [$^2H_8$]SO-Hb [12].

2.2. Elimination of SO from blood in vitro

The first-order elimination rate constant (designated k_e) for SO in mice and human blood was estimated essentially as described in Rappaport et al. [21]. Thirty μl of 10 mM SO in isotonic saline was mixed with 7.5 ml whole human blood (stored at 4°C for 24 h prior to use) at 37°C to give an initial concentration of 40 μM. Aliquots of 1 ml were taken at 0, 5, 10, 15, 20, 30, 60, 90, and 120 min and immediately extracted with 3 ml hexane containing 10 μM [$^2H_8$]SO as the internal standard. After freezing, thawing, and centrifuging the sample, the hexane layer was removed and dried with $MgSO_4$. The hexane layer was injected on-column onto a HP 5890 series II gas chromatograph coupled to a HP 5970 mass selective detector. Helium (4 psi) was used as the carrier gas. The chromatographic separation was carried out on a DB-5MS fused silica capillary column (30 m, 0.25 mm i.d., 0.1 μm phase thickness, Alltech Associates). The interface was kept at 270°C and the oven temperature was programmed at 7°C per min from 60°C to 100°C, followed by an increase of 20°C per min to 200°C. The injector temperature was maintained at 3°C higher than the oven temperature. The mass spectrometer was operated in the electron impact mode with an ionization energy of 70 eV. The peak areas of the ions m/z 91, 119 and 120 for SO and the ions m/z 98, 126 and 128 for [$^2H_8$]SO were obtained by selected ion monitoring (SIM). The concentration of SO in blood was determined by comparing the average peak area ratio of SO to [$^2H_8$]SO to an established calibration curve.

This experiment was repeated using blood from NMRI mice, except that the starting concentration of SO was 30 μM.

2.3. Modification of blood with SO in vitro

Human, mouse and rat blood were incubated in vitro with increasing levels of SO in order to estimate the site-specific second-order reaction rate constants ($k_{SO\text{-}Y}$) as described below. 0.25 to 32 μl of 126 mM SO in isotonic saline was added to 4 ml aliquots of human blood to give final concentrations of 0, 10, 30, 100, 300, and

1000 μM. 0.25 to 76 μl of 126 mM SO in isotonic saline was added to 3–4 ml aliquots of NMRI mouse blood to achieve final concentrations of 0, 7.9, 24, 79, 100, 300, 600, 790, 1000, 2000, and 2400 μM. SO (0–7.5 mM) in 0.5 ml of isotonic saline was added to 12 ml aliquots of Sprague–Dawley rat blood resulting in final concentrations of 0, 10, 30, 100, and 300 μM as described in Rappaport et al. [21]. All blood samples were incubated for 2 h at 37°C. Hb adducts were determined by analyzing SO-bound cysteine, N-terminal valine and carboxylic acid residues and were converted from units of nmol adduct/g Hb to μM, assuming that the concentration of Hb is 152, 135 and 153 mg/ml in human, mouse and rat blood, respectively [23–25].

2.4. Animal experiments

Styrene was mixed with dimethylsulfoxide and corn oil and administered i.p. to 30 male NMRI mice (25–30 g) at 0, 0.86, 1.78, 3.51, and 7.43 mmol/kg body weight as described in Pauwels et al. [15]. However, for this study, Hb adducts were not determined at the highest dosage. The animals were anesthetized with Nembutal (Sanofi, Belgium) 3 h post administration and the blood was removed by cardiac puncture. Ten male (average weight 563 g) and ten female (average weight 360 g) Sprague–Dawley rats were dosed with 0, 0.5, 1.0, and 3.0 mmol styrene/kg body weight or 0, 0.1, 0.3, and 1.0 mmol SO/kg body weight in corn oil as described in Rappaport et al. [21]. The animals were anesthetized with methoxyflurane and the blood was removed by cardiac puncture with a heparinized syringe 24 h after administration.

2.5. Isolation of globin

Red blood cells were separated from plasma by centrifugation at $800 \times g$ for 10 min and were washed with three volumes of isotonic saline prior to lysis with one volume of water. Mouse and human globin was purified by the method of Mowrer et al. [26] and the mouse globin was then pooled by dosage group prior to adduct analysis. Briefly, acidic propanol was added to the red blood cells and, following centrifugation, the globin was precipitated by the addition of ethyl acetate. Rat globin was purified as described in Rappaport et al. [21]. The red blood cell membranes were removed by centrifugation at $30\,000 \times g$, the resulting Hb solution was desalted via Sephadex chromatography, and the globin was precipitated into cold acidic acetone. The globin was stored at -20°C for up to 3 years prior to analysis.

2.6. Analysis of cysteine and carboxylic acid adducts

SO-COOH and SO-cys Hb adducts were analyzed as described in Yeowell-O'Connell et al. [19]. A sample of 10 mg globin was combined with 10 μg 4-methyl-SO-Hb and digested for 4 h with protease XIV. Following the addition of NaOH, the solutions were heated to 100°C to release COOH-bound SO as SG,

which was extracted into ethyl acetate. The solvent was then removed under nitrogen and the analyte (SG) and internal standard (4-methyl-SG) were derivatized with pentafluorobenzoyl (PFB) chloride. To the remaining protein solution was added 3-phenylpropanol (free internal standard) and Raney nickel to release the cysteine-bound adducts as 1-phenylethanol (1-PE) and 2-phenylethanol (2-PE). After extracting the cleavage products with diethyl ether, the solvent was removed and the analytes (1-PE and 2-PE) and internal standards (4-methyl-2-PE and 3-phenylpropanol) were derivatized with PFB chloride prior to quantitation by gas chromatography-mass spectrometry (GC-MS).

2.7. Analysis of N-terminal valine adducts

SO-val Hb adducts were analyzed using the method of Törnqvist et al. [27] as described in Pauwels et al. [15]. A sample of 50 mg globin and 10 ml internal standard (containing 1.2 mg globin/ml and 1.73 nmol *N*-(2-hydroxy-2-[$^2H_8$]phenylethyl)valine/mg [$^2H_8$]SO-Hb) were reacted with pentafluorophenyl isothiocyanate under basic conditions. The resulting thiohydantoin derivatives were extracted into diethyl ether, washed with water and $NaHCO_3$ and redissolved in toluene for analysis by GC-MS.

2.8. GC-MS of adducts

The pentafluorobenzoate derivatives of SO-COOH and SO-cys adducts were analyzed by GC-MS in the negative ion chemical ionization mode using a HP 5989A MS engine coupled to a HP 5890 series II gas chromatograph. The GC column and instrument conditions were the same as described in Yeowell-O'Connell et al. [19]. The SIM ions monitored were m/z 316 (for the PFB derivatives of SG, 1-PE and 2-PE) and 330 (for the PFB derivatives of 4-methyl-SG, 4-methyl-2-PE and 3-phenylpropanol). The pentafluorophenylthiohydantoin derivative of the SO-val adduct was analyzed by GC-MS in the electron impact mode using an HP 5970 mass selective detector coupled to an HP 5890 series II gas chromatograph. The column and instrument conditions were the same as those described by Pauwels et al. [15]. The analyte and deuterated internal standard were quantified by SIM of the ions m/z 325 and 326, respectively.

2.9. Estimation of the second-order reaction rate constants

The second-order reaction rate constant, $k_{\text{SO-Y}}$ (in L (mol Hb)$^{-1}$ h^{-1}), indicates the relative affinity of a site on a protein for the reactive metabolite of interest and is defined as follows [21,28]

$$k_{\text{SO-Y}} = \frac{[\text{SO-Y}]k_e}{[\text{Hb}][\text{SO}]_0(1 - e^{-k_e t})} = \frac{\beta_Y k_e}{[\text{Hb}](1 - e^{-k_e t})} \quad (1)$$

where [SO-Y] (in mM) is the concentration of adducts resulting from the reaction of SO with cysteine, valine, or COOH residues in Hb, k_e (in h^{-1}) is the pseudo

first-order elimination rate of SO from blood in vitro, t (in h) is the amount of time SO was incubated with whole blood, β_Y (in units of μM SO-Hb/μM SO) is the slope of the regression of the concentration of each adduct on the initial concentration of SO (designated $[SO]_0$), and [Hb] is the concentration of Hb (2.1×10^{-3} and 2.3×10^{-3} M for mouse and rat blood, respectively [24,25]).

2.10. Estimation of SO blood dose from Hb adduct levels

Integrated dose (or area under the curve, AUC, in units of mM-h) was predicted using the following relationship [29,30]:

$$\text{Dose} = \frac{[\text{SO-Y}]}{k_{\text{SO-Y}}[\text{Hb}]} \tag{2}$$

where [SO-Y], k_{SO-Y} and [Hb] are defined above. The dose of SO per unit dosage of styrene or SO was calculated based upon the adduct levels determined for each binding site in mice and rats dosed with styrene and in rats dosed with SO.

2.11. Estimation of SO blood dose using the Csanády model

The SO concentration in the blood of mice and rats after i.p. administration of styrene or SO was predicted using the physiologically-based pharmacokinetic (PBPK) model of Csanády et al. [31] as described in Pauwels et al. [14]. The parameters of the model were adjusted to reflect the experimental conditions used for the in vivo experiments in this study. The integration of the SO concentration in the blood over the duration of the experiment provided a prediction of the AUC of SO in blood, which was then compared to the dose, based upon the levels of Hb adducts as defined by Eq. (2).

2.12. Data analysis

All statistical analyses were performed using SAS-PC (SAS Institute, Cary, NC), with a P-value ≤ 0.05 indicating statistical significance. All comparisons were made using 2-tailed z-tests. The appropriateness of unweighted least squares regression was evaluated visually from plots of the residuals. For rats dosed with styrene or SO, the assumption of homogeneous variance was not satisfied; hence, weighted least squares regression was used to estimate the slopes and intercepts of each adduct regressed upon administered dosage [32]. The weight was set equal to the inverse of the variance of adduct levels estimated at each dosage level. For the mice dosed with styrene, the Hb samples at each dosage level were pooled prior to adduct analysis; consequently, the variance could not be estimated and unweighted least squares regression was used.

3. Results

3.1. Elimination of SO from blood in vitro

The elimination rate constants and corresponding half-times for SO in human, NMRI mouse, and Sprague–Dawley rat blood at 37°C are given in Table 1. While the half-times of SO in mouse and rat blood were fairly similar (20–22 min), that in human blood was about twice as long (42 min).

3.2. Modification of blood with SO in vitro

The formation of SO-Hb adducts in vitro was investigated by incubating human, NMRI mouse and Sprague–Dawley rat blood with initial SO concentrations ($[SO]_0$) of 0–1000, 0–2400 and 0–300 μM, respectively. The results from the regression of Hb adducts ([SO-Y], where Y represents the cysteine, N-terminal valine or carboxylic acid residues of Hb) on $[SO]_0$ are given in Table 2. R^2 values ranged from 0.897–0.998 for the linear ranges of $[SO]_0$ noted. Although the linear ranges were expected to be the same for all adducts within each species, SO-COOH and SO-val adducts were disproportionately higher in mouse blood incubated with values of $[SO]_0$ above 1000 μM while SO-cys (both 1-PE and 2-PE) adducts increased linearly with $[SO]_0$ up to 2500 μM. Such trends were not noted for incubations carried out in human or rat blood, probably due to the lower $[SO]_0$ concentrations used.

Estimates of the slopes of these linear relationships (designated β_Y), coupled with the first-order elimination rate constants given in Table 1, were used to estimate the rates of reaction of SO with the different nucleophilic sites on human, mouse and rat Hb according to Eq. (1). Estimates of these second-order reaction rate constants ($k_{SO\text{-}Y}$) are given in Table 3. Note that the S.E. of $k_{SO\text{-}Y}$ represents the combined errors associated with the estimation of both k_e and β_Y. While the rate constants for cysteine adducts (for binding through either the α or β carbon of SO) varied dramatically between species, the constants for reactions of valine adducts and carboxylic acid residues were more similar. However, in all cases, each site-specific $k_{SO\text{-}Y}$ was statistically different across species ($P < 0.001$), except for $k_{SO\text{-}COOH}$ in

Table 1
First-order elimination rate constants and half-times for SO in blood from humans, NMRI mice and Sprague–Dawley rats

Species	k_e (h^{-1})	S.E.[a] (h^{-1})	N	$T_{1/2}$ (h)	$[SO]_0$ (μM)
Human[b]	0.99	0.087	4	0.70	30–40
Mouse	2.13	0.325	2	0.33	30
Rat[c]	1.90	0.064	3	0.36	30

[a] S.E. of N determinations.
[b] Data are combined with the two determinations from Rappaport et al. [21].
[c] Data are from Rappaport et al. [21].

Table 2
Linear regression[a] of Hb adduct formation on $[SO]_0$ for blood from humans, NMRI mice and Sprague–Dawley rats modified with styrene oxide in vitro

Species	Estimated parameter	Cys (2-PE)	Cys (1-PE)	Val	COOH
Human	β_Y^b (S.E.)	3.9 (0.10)	0.15 (0.01)	3.5 (0.07)	6.8 (0.17)
	β_0^c (S.E.)	101 (44)	14 (5.6)	−16 (31)	65 (74)
	Linear range (μM)	0–1000	0–1000	0–1000	0–1000
Mouse	β_Y (S.E.)	9.9 (1.0)	2.0 (0.20)	0.74 (0.05)	1.8 (0.23)
	β_0 (S.E.)	−767 (1076)	159 (208)	−35 (23)	60 (111)
	Linear range (μM)	0–2400	0–2400	0–1000	0–1000
Rat	β_Y (S.E.)	157 (4.3)	24 (1.3)	0.34 (0.02)	2.8 (0.06)
	β_0 (S.E.)	254 (557)	−111 (175)	−2.0 (2.3)	−12.5 (7.6)
	Linear range (μM)	0–300	0–300	0–300	0–300

[a] R^2 values ranged from 0.897–0.998.
[b] β_Y is the slope of the linear relationship with units of nM SO-Hb/μM SO.
[c] β_o is the intercept with units of nM SO-Hb.

mice vs. rats. The rate constants were also statistically different within species, except for $k_{\text{SO-cys (1-PE)}}$ and $k_{\text{SO-COOH}}$ in mice and $k_{\text{SO-cys (2-PE)}}$ and $k_{\text{SO-val}}$ in humans.

3.3. Formation of SO-Hb adducts in vivo

The formation of SO-Hb adducts was investigated in NMRI mice administered ip 0.86–3.51 mmol styrene/kg body weight by analyzing Hb for SO-bound cysteine, N-terminal valine and carboxylic acid residues. Fig. 1 shows Hb adduct levels (in nmol adduct/g Hb) at each site plotted vs. styrene dosage. The results of the linear regressions are given in Table 4.

Table 3
Second order rate constants for the reaction of styrene oxide with cysteine, N-terminal valine and carboxylic acid residues of Hb in human, mouse and rat blood

	Estimated rate constant[a] (L (mol Hb)$^{-1}$ h^{-1})			
	$k_{\text{SO-cys (2-PE)}}^b$ (S.E.)	$k_{\text{SO-cys (1-PE)}}$ (S.E.)	$k_{\text{SO-val}}$ (S.E.)	$k_{\text{SO-COOH}}$ (S.E.)
Human	2.04 (0.19)	0.078 (0.010)	1.82 (0.16)	3.55 (0.32)
Mouse	10.7 (2.0)	2.16 (0.39)	0.80 (0.13)	1.94 (0.39)
Rat	133 (5.8)	20.4 (1.3)	0.29 (0.017)	2.37 (0.094)

[a] All of the site-specific reaction rate constants were statistically different ($P<0.001$) across species except for $k_{\text{SO-COOH}}$ in mice vs. rats ($P=0.14$). The rate constants were also different within species, except for $k_{\text{SO-cys (1-PE)}}$ vs. $k_{\text{SO-COOH}}$ in mice ($P=0.35$) and $k_{\text{SO-cys (2-PE)}}$ vs. $k_{\text{SO-val}}$ in humans ($P=0.19$).
[b] Cysteine can attack SO through either the α or β carbon; following reaction of the adducts with Raney Ni, 2-PE and 1-PE are generated, respectively.

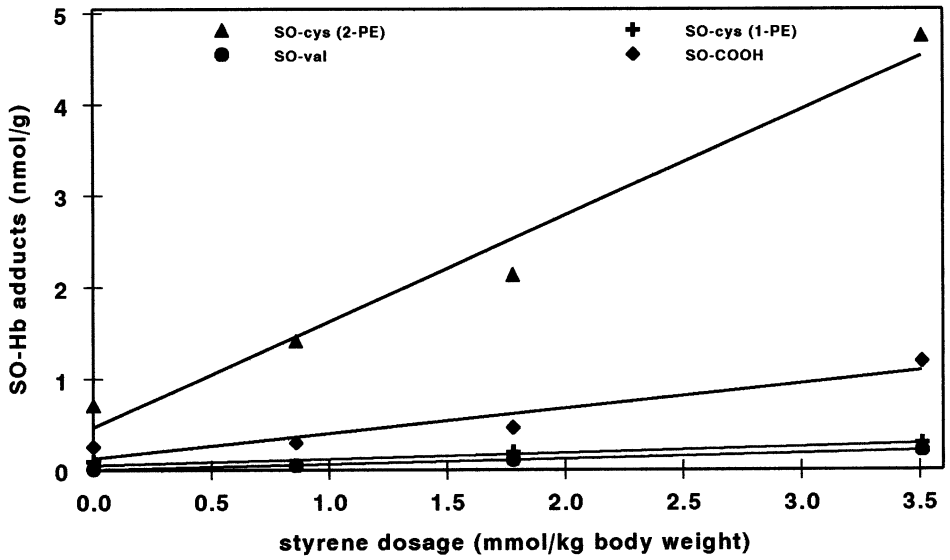

Fig. 1. Formation of SO-Hb adducts following i.p. administration of styrene to NMRI mice in vivo by analysis of SO-bound cysteine, N-terminal valine and carboxylic acid residues. Blood was collected 3 h after dosing. Each point represents a single determination of pooled Hb. The SO-valine data are taken from Pauwels et al. [15]. The slopes and intercepts of the linear regression are given in Table 4.

The formation of site-specific SO-Hb adducts was also investigated in Sprague–Dawley rats administered i.p. 0.5–3.0 mmol styrene/kg body weight or 0.1–1.0 mmol SO/kg body weight. The site-specific Hb adduct levels were plotted vs. styrene (Fig. 2A) or SO dosage (Fig. 2B) and the results of the weighted least squares regressions are given in Table 5. For both mice and rats, the levels of SO-cysteine (2-PE) adducts were the highest and valine adducts were the lowest.

Table 4
Linear regression of Hb adducts on dosage of styrene administered to NMRI mice in vivo

Site	β_Y^b (S.E.)	β_0^c (S.E.)	R^2	Linear range (mmol agent/kg body wt)
Cys (2-PE)	1160 (138)	470 (278)	0.972	0–3.51
Cys (1-PE)	67 (21)	55 (43)	0.835	0–3.51
Val[a]	60 (2.4)	4.8 (4.8)	0.997	0–3.51
COOH	276 (63)	128 (127)	0.906	0–3.51

[a] Data are from Pauwels et al. [15].
[b] β_Y is the slope of the linear relationship with units of pmol adduct/g globin (mmol agent/kg body weight)$^{-1}$.
[c] β_o is the intercept with units of pmol adduct/g globin.

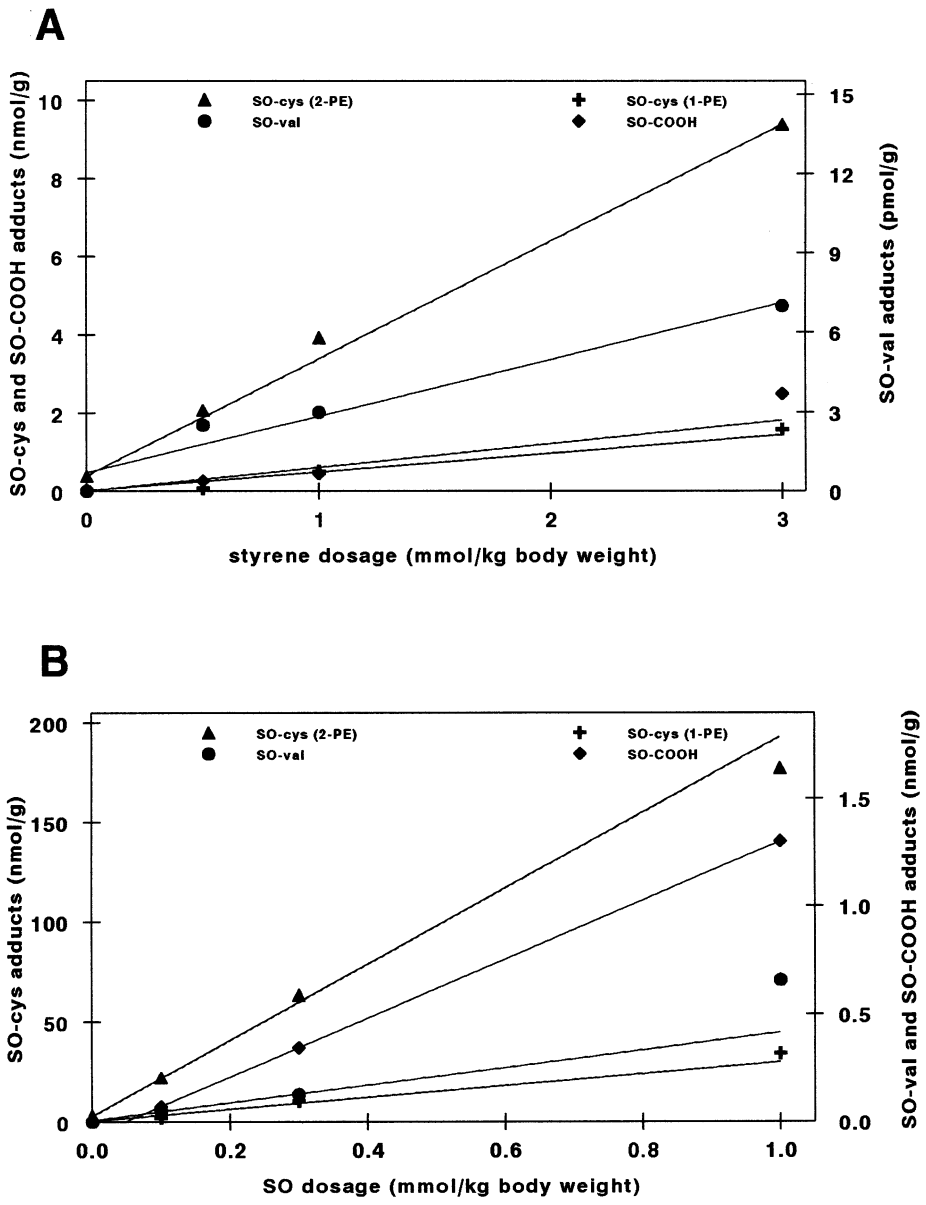

Fig. 2. Formation of SO-Hb adducts following i.p. administration of (A) styrene and (B) SO to Sprague–Dawley rats in vivo by analysis of SO-bound cysteine, N-terminal valine and carboxylic acid residues. Blood was collected 24 h after dosing. Each point represents the mean of two to three animals per dosage group. Note the different axes. The slopes and intercepts obtained from weighted least squares regression are given in Table 5. The SO-val data in rats dosed with styrene should be interpreted cautiously as most points are at or below to the limit of detection (10 pmol/g).

Table 5
Linear regression[a] of Hb adducts on dosage of styrene or styrene oxide administered to Sprague–Dawley rats in vivo

Agent	Site	β_Y^b (S.E.)	β_0^c (S.E.)	R^2	Linear range (mmol agent/kg body wt)
Styrene	Cys (2-PE)	3010 (139)	395 (143)	0.983	0–3
	Cys (1-PE)	488 (160)	14 (6.2)	0.999	0–3
	Val	2.1 (0.70)	0.73 (1.3)	0.540	0–3
	COOH	607 (121)	3.0 (3.0)	0.758	0–3
SO	Cys (2-PE)	$1.90\ (0.204) \times 10^5$	2750 (400)	0.916	0–1
	Cys (1-PE)	$3.08\ (0.380) \times 10^4$	310 (140)	0.891	0–1
	Val	437 (56)	1.4 (6.2)	0.996	0–1
	COOH	1240 (240)	−26 (46)	0.947	0–1

[a] Weighted least squares regression with weight = 1/variance of the level of adducts at each dosage.
[b] β_Y is the slope of the linear relationship with units of pmol adduct/g globin (mmol agent/kg body weight)$^{-1}$.
[c] β_o is the intercept with units of pmol adduct/g globin.

3.4. Blood dose of SO per dosage of styrene or SO

Using Eq. (2), the SO blood dose (or AUC) per unit dosage [mM SO-h (mmol styrene or SO/kg body weight)$^{-1}$] was calculated for each adduct in both mice and rats based upon the estimated site-specific second-order reaction rate constants (Table 3) and regression of adduct levels on dosage of styrene or SO (Tables 4 and 5). These estimates of dose/dosage are given in Table 6, with an asterix designating the ones that are significantly different from those based upon the levels of SO-cys (2-PE) adducts. Note that the S.E. of the estimated dose per unit dosage encompasses the error associated with the estimation of the slope of the regression of adduct level on administered dosage as well as that for $k_{SO\text{-}Y}$.

The site-specific estimates of predicted SO dose/dosage of styrene for SO-cys (2-PE), SO- val and SO-COOH obtained for mice were not statistically different ($P \geq 0.1$). However, the slope based upon SO-cys (1-PE) adducts was statistically

Table 6
Blood dose of SO per dosage styrene administered to NMRI mice and Sprague–Dawley rats, and per dosage styrene oxide administered to Sprague–Dawley rats

Species	Agent	Blood dose of SO per dosage styrene or SO [μM SO-h (mmol agent/kg body weight)$^{-1}$] (S.E.)			
		Cys (2-PE)	Cys (1-PE)	Val	COOH
Mouse	Styrene	7.3 (1.6)	2.1 (0.76)*	5.0 (0.84)	9.6 (2.9)
Rat	Styrene	1.5 (0.10)	1.6 (0.54)	0.49 (0.16)*	17 (3.5)*
Rat	SO	95 (11)	101 (14)	100 (14)	35 (6.7)*

* Significantly different from that determined using SO-cys (2-PE) adducts with $P < 0.05$.

different from those of all the other adducts ($P \leq 0.01$). This discrepancy may be due to non-linearity of Hb adduct formation at higher dosages of styrene. If the linear range was taken to be 0–1.78 mmol styrene/kg body weight for both cysteine adducts, no differences in dose/dosage were observed. However, this experiment did not have enough data points to firmly establish the linear range for Hb adduct formation in mice dosed with styrene.

For rats dosed with styrene, the estimates for the cysteine adducts (1-PE and 2-PE) were not statistically different ($P = 0.7$) although those for valine and COOH adducts were different from both cysteine adducts ($P \leq 0.006$). However, the dose estimated using valine adducts should be considered very approximate, because most adduct levels were at or below the limit of detection (10 pmol/g globin). Surprisingly, the dose estimated based upon COOH adducts was much higher than that predicted using cysteine or valine adducts.

Concerning the rats dosed with SO, the estimated dose/dosage of SO for cysteine (1-PE and 2-PE) and valine adducts were not statistically different ($P \geq 0.8$), while that based upon COOH adducts was again not in agreement with the other adducts ($P \leq 0.03$); however in this case, the estimated dose was much lower than that predicted based upon the other adducts. This underestimate of the SO dose may be due to known instability of SO-COOH adducts in vivo [13]. The discrepancy in the ability of SO-COOH adducts to be used to estimate SO dose in mice versus rats may be related to the fact that the globin from these two species was isolated using somewhat different procedures and that SO-COOH adducts have been reported to degrade during storage of acid-precipitated globin [13].

4. Discussion

As noted in Section 1, SO can undergo reaction with nucleophilic sites in Hb through either the α or β carbon. Cysteine can form SO-cys adducts at both positions which, following reaction with Raney nickel, gives rise to 2-PE and 1-PE, respectively [19]. However, SO-valine appears to be formed only via reaction through the β carbon, giving rise to *N*-(2-hydroxy-2-phenylethyl)valine [12]. SO also reacts with carboxylic acid residues to yield two phenylhydroxyethyl esters, both of which are readily hydrolyzed under basic conditions to give SG [19].

4.1. Second-order reaction rate constants

The site-specific second-order reaction rate constants (Table 3) for the reaction of SO with Hb depends upon the intrinsic reactivity of SO at a particular locus as well as the accessibility and number of loci of a given type in the Hb molecule. As such, the second-order reaction rate constants (Table 3) showed that for human Hb, binding was very similar for all three sites of reaction (2.12, 1.82, and 3.55 L (mol Hb)$^{-1}$ h^{-1} for cysteine ($k_{\text{SO-cys (2-PE)}} + k_{\text{SO-cys (1-PE)}}$), valine, and COOH residues, respectively). These values corresponded well to results based upon [^{14}C]SO-binding in human Hb modified with SO in vitro; cysteine binding accounted for $\sim 6\%$ of

the total SO bound to Hb [21], N-terminal valine binding represented ~ 3–5% [9,33] and carboxylic acid binding represented $\sim 15\%$ [18]. Although in vitro experiments involving individual amino acids and isolated human blood proteins indicated that cysteine residues were the most reactive towards SO [34], work by Kaur et al. [35] with human Hb modified in vitro indicated that the highly accessible β-His-143 and α-His-20 residues were the major sites of SO modification, while relatively little SO reacted with β-Cys-93 (the most reactive cysteine residue in human Hb).

However, in rats, higher levels of cysteine binding were expected than in humans due to the presence of a cysteine residue at position β-125 [36] in addition to β-Cys-93, which is common to rat, human and mouse Hb. In mice, some strains (BALB/c and mus musculus) also contain an extra cysteine at position β-13, although others (C57B1, SWR and NB) do not (discussed in [37]). Whereas the calculated rate constant (153 L (mol Hb)$^{-1}$ h^{-1}) for SO-cys ($k_{\text{so-cys (1-PE)}} + k_{\text{so-cys (2-PE)}}$) in Sprague–Dawley rats clearly reflected the existence of the additional cysteine, the corresponding rate constant (10.7 L (mol Hb)$^{-1}$ h^{-1}) for NMRI mice was still large relative to human Hb (2.04 L (mol Hb)$^{-1}$ h^{-1}), and may indicate either the presence of an additional cysteine residue or some other difference in the Hb structure.

In contrast to SO-cys formation, the rate of reaction of SO with valine and COOH residues in Hb was expected to be much more consistent across species. Indeed, our results showed only 5-fold variation for reaction of SO with valine (1.82, 0.80, 0.29 L (mol Hb)$^{-1}$ h^{-1}, for humans, mice and rats, respectively) and even less variation for COOH residues (3.55, 1.94, 2.37 L (mol Hb)$^{-1}$ h^{-1}, respectively). Although the species-specific rate constants for reaction of valine with SO are much more similar than those for cysteine, our results clearly point to significant differences between species that have not been recognized heretofore. We, therefore, encourage others to estimate the second-order reaction rate constants for reactions of all electrophiles with N-terminal valine prior to making inferences about the tissue dose.

The only reported comparisons of second-order rate constants for electrophiles with different sites on the same protein or with the same site in different species involved experiments with ethylene oxide and propylene oxide. The reported rate constants for the reaction of ethylene oxide with the N-terminal valine in human, mouse and rat Hb (assuming a MW of 66 000 g/mol Hb) were 3.0, 2.1 and 3.0 L (mol Hb)$^{-1}$ h^{-1}, respectively [38], while those for the reaction of propylene oxide with valine were 1.7, 0.92 and 1.5 L (mol Hb)$^{-1}$ h^{-1}, respectively [39]. These compounds showed less inter-species variation than that observed for the reaction of SO with valine. However, for the reaction of ethylene oxide with cysteine the inter-species differences (0.40, 4.6, 66 L (mol Hb)$^{-1}$ h^{-1} for human, mouse and rat Hb, respectively) [38] were even more pronounced than those observed herein for SO. Overall, the general trends in the site-specific and species-specific reactivity with Hb are very similar for SO, ethylene oxide and propylene oxide.

Assuming that the cysteine adducts resulting from reaction through the α- and β-carbons of SO are equally stable, then the ratios of the slopes of the regressions

of SO-cys adducts (2-PE and 1-PE) on $[SO]_0$ in vitro suggest that SO is about 5.0 and 6.5 times more likely to react through the α carbon than through the β carbon in mice and rats, respectively. These ratios were 2–4 times higher than other published results obtained in vitro. For the reaction of SO with the cysteine residue in glutathione, *N*-acetylcysteine and free cysteine, the reported α-to-β-binding ratios were 1.5, 1.9 and 2, respectively [34,40,41]. For mice dosed i.p. with styrene, the calculated α-to-β-binding ratio of ~ 16 (from Table 4) was even higher; however, the ratios in rats dosed with styrene or SO (Table 5; 5.9 and 5.1, respectively) were very similar to that reported in vitro.

4.2. Comparison of the predicted blood dose of SO with model results

The blood dose of SO, as predicted from SO-Hb adducts, can be compared to the SO AUC estimated by directly measuring SO in the blood of rodents dosed with styrene or SO. However, since we did not make these measurements in the animals used in this experiment and the literature values of SO AUC were generally determined under somewhat different experimental conditions than those used in this study, we used the PBPK model for styrene and SO developed by Csanády et al. [31] in order to predict the SO AUC for rats and mice under the experimental conditions applied to the animals in this study. Since good agreement has been observed between experimentally measured values for the integrated dose of SO and the values obtained from this PBPK model, these values should reflect reasonable estimates of SO AUC [31].

For a 25 g mouse dosed i.p. with 1 mmol styrene/kg body weight, the predicted AUC for SO after 3 h was 6.4 μM-h, which was very close to our estimate of 7.3 μM-h per unit dosage of styrene (Table 6) based on SO-cys (2-PE) adducts. For a 462 g rat dosed i.p. with 1 mmol styrene/kg body weight, the predicted AUC of SO after 24 h was 5.3 μM/h, which was 3.5 times higher than our estimate of 1.5 μM-h per unit dosage of styrene (Table 6) based on SO-cys (2-PE) adducts. Finally, for a 462 g rat dosed i.p. with 1 mmol SO/kg body weight, the predicted AUC for SO after 24 h was 33 μM h, which was about one-third of our estimate of 95 μM-h per (mmol SO/kg body weight) based upon SO-cys (2-PE) adducts.

These values of SO dose (or AUC) per unit dosage of styrene or SO were used to estimate the fraction of SO which reaches the red blood cell following the administration of styrene to rats [21]. Although we calculated (using SO-cys (2-PE) adducts) that this fraction was 1.5 μM-h (mmol styrene/kg body weight)$^{-1}$/95 μM-h (mmol SO/kg body weight)$^{-1} = 1.6\%$, calculations based upon the PBPK model gave a 10-fold higher estimate (i.e. 5.3 μM-h (mmol styrene/kg body weight)$^{-1}$/33 μM-h (mmol SO/kg body weight)$^{-1} = 16\%$). The discrepancy between the SO AUC predicted using the PBPK model and that estimated by SO-Hb adducts may be due to the fact that the rats used in this experiment were much older and larger than those used to develop the model of Csanády et al. [31]. Consequently, the different metabolizing capabilities of the two groups of rats may have contributed to the different estimates of the SO dose [42–44].

As expected, the Hb adduct data indicated that much more SO was available to the blood of mice than rats dosed with styrene; i.e. based upon SO-cys (2-PE) adducts, the relative blood dose of SO per unit dosage of styrene was 7.3 μM-h (mmol styrene/kg body weight)$^{-1}$/1.5 μM-h (mmol styrene/kg body weight)$^{-1}$ = five times higher in mice than in rats. Overall, we regard the 2–4-fold agreement between SO dose predicted from Hb adducts and those estimated from the PBPK model as quite good considering the many possible sources of error.

4.3. Comparison with other studies of SO-Hb adducts in rodents

Earlier work [21] had reported SO-cys adducts (only 2-PE) in the Sprague–Dawley rats dosed with styrene or SO, using a slightly different version of the Raney nickel procedure which could not detect 1-PE and which did not include the hydrolysis step to release SO-COOH adducts. The inclusion of this hydrolysis step resulted in $\sim$1.5-fold higher estimates of SO-cys (2-PE) adducts. However, when the previous assay [21] was used to estimate the second-order reaction rate constant as well as to calculate the blood dose in rats dosed in vivo, the estimated SO dose per unit of dosage was not significantly different for rats dosed with either styrene (2.02 ± 0.46 vs. 1.5 ± 0.10 μM-h (mmol styrene/kg body weight)$^{-1}$, $P = 0.41$) or SO (109 ± 13 vs. 95 ± 11 μM-h (mmol SO/kg body weight)$^{-1}$, $P = 0.27$).

The levels of SO-val adducts can be compared to the work of Osterman-Golkar et al. [13]. At 1 mmol styrene/kg body weight in NMRI mice dosed i.p., Osterman-Golkar et al. [13] reported 75 pmol SO-val/g globin, which is similar to what we observed [60 ± 2.4 (S.E.) pmol/g (mmol/kg)$^{-1}$] (Table 4). For Sprague–Dawley rats dosed i.p. with styrene or SO, they reported 20 pmol/g (mmol styrene/kg)$^{-1}$ and 130 pmol/g (mmol SO/kg)$^{-1}$, in contrast to 2.1 ± 0.70 pmol/g per (mmol styrene/kg) and 437 ± 56 pmol/g (mmol SO/kg)$^{-1}$ (Table 5) observed in this investigation. The slopes of the dose-response curves reported by Osterman-Golkar et al. [13] were estimated by eye and are heavily dependent upon the linear range chosen. Furthermore, the rats used in their experiments were much smaller (200–280 g) and younger than those in this investigation. Consequently, the different metabolizing capabilities of the two groups of rats could have contributed to the different estimates of the slopes [42–44].

The levels of SO-COOH adducts are very similar to that reported by Sepai et al. [18] who also used base hydrolysis to release carboxylic acid-bound SO from Sprague–Dawley rats dosed i.p. with 0.083–0.83 mmol SO/kg body weight. They reported 1070 pmol/g per mmol SO/kg body weight, which is not significantly different from the 1240 ± 240 pmol/g per mmol SO/kg body weight (Table 5) observed herein.

5. Conclusions

Levels of SO-cys, SO-val and SO-COOH in Hb all showed increasing linear trends with dosage of styrene or SO administered to mice and rats. The SO blood

dose predicted from valine or cysteine adducts was not inconsistent with the AUC of SO predicted from the model of Csanády et al. [31]. This lends credence to the use of Hb adducts as dosimeters. While SO-cys and SO-val adducts gave similar estimates of the SO blood dose, SO-COOH adducts gave very different results, probably due to the known instability of SO-COOH adducts. Consequently, the inability to detect dose-related increases of SO-COOH adducts in human biomonitoring studies [18,19] is not surprising. Although SO-cys and SO-val have both produced consistent results in animal studies, they have shown inconsistent results in human studies. Part of the difficulty in applying SO-val may relate to the limit of detection for SO-val (10 pmol/g globin), which would make SO-val suitable for use only at high levels of styrene exposure [11,12]. Although the limit of detection for SO-cys (2-PE) (1 pmol/g) allows for detection of lower Hb adduct levels, the high levels of background adducts detected in unexposed individuals also makes biomonitoring at low styrene exposures difficult [19,22].

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